ANALYSE DE LA QUALITÉ MICROBIOLOGIQUE ET DE LA STABILITÉ D'UNE PRODUCTION INDUSTRIELLE DE CHOUCROUTE

par

Yanick Bisaillon

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FACULTÉ DES SCIENCES
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le jury a accepté le mémoire de Monsieur Yanick Bisaillon
dans sa version finale.

Membres du jury

Professeure Carole Beaulieu
Directrice de recherche
Département de biologie

Tony Savard
Codirecteur de recherche
Agriculture et Agroalimentaire Canada

Professeur Sébastien Roy
Évaluateur interne
Département de biologie

Professeur Luc Gaudreau
Président-rapporteur
Département de biologie

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Le but de ce projet était d'investiguer la variabilité observée dans les fermentations industrielles de choucroute spontanées en comparaison avec celles utilisant un ferment mixte. Le ferment BLAC I utilisé dans cette étude a été conçu initialement par Gardner et al. (2001) pour standardiser la production de légumes fermentés. D'abord, la comparaison d'une fermentation spontanée à celle avec le ferment a permis de déterminer si la microflore indigène du chou, ayant évoluée durant les 20 dernières années, serait plus compétitive envers le ferment qu'au moment de sa conception. L'impact de la mise à l'échelle a ensuite été évalué en comparant des fermentations réalisées à l'échelle industrielle à celles réalisées en laboratoire. Par la suite, l'utilisation de choux biologiques ou conventionnels a été comparée, puisque la culture de légumes biologiques a beaucoup évoluée au cours des dernières années, notamment avec le développement de plusieurs pesticides biologiques pouvant influencer la microflore indigène du légume, l'hypothèse d'un impact de ces pesticides sur le développement des fermentations, qu'elles soient spontanées ou non, avait été avancée.

Une production de choucroute à l'échelle industrielle en cuves de 400 kg a donc été suivie en usine en comparaison avec des seaux de 20 kg produits en laboratoire pour chaque condition testée et ce à partir du même lot de choux. Seule l'échelle de production présentait donc une variable. L'évolution des bactéries fermentaires, levures, flores totales et coliformes a été suivie sur une durée de 180 jours. L'analyse de ces fermentations a permis de déterminer que le ferment est toujours efficace, mais qu'une mortalité des bactéries lactiques du ferment survient lors de la phase de maturation. Cette mortalité, bien que présente en ferment et en spontané, était accélérée dans le cas où un ferment était utilisé, et ce même si les comptes finaux étaient indépendants de l'utilisation de ferment. Un point important à souligner est que le mode de production du chou, qu'il soit biologique ou conventionnel, n'influencerait pas la qualité de la fermentation lorsqu'un ferment est utilisé. Aussi, la survie et la croissance de

levures acido-résistantes a été observée à la surface des cuves de fermentation et ceci aussi bien avec que sans ferment mais les levures n'ont pas été détectées en seaux de 20 kg.

Par conséquent, le deuxième volet de cette étude consistait à détecter la présence de biofilm fongique à la surface des cuves de fermentation industrielles qui sont composées d'acier inoxydable. Nous avons donc procédé au prélèvement, à l'identification des levures isolées en phase de fermentation et de maturation des choucroutes puis à l'évaluation de leur capacité à former un biofilm à l'échelle laboratoire. Les levures qui contaminent les productions de légumes fermentés représentent un problème majeur en industrie parce qu'elles peuvent causer une fermentation secondaire et ainsi remonter le pH en métabolisant les acides organiques, ce qui diminue les barrières de contrôle des pathogènes dans le produit final. Toutefois le principal problème repose sur l'utilisation des sucres résiduels pour produire du gaz, causant des problèmes d'emballage et de durée de vie du produit au niveau industriel.

La présence de biofilms a bel et bien été confirmée dans cette étude. Grâce à la méthode d'échantillonnage il a été possible de prélever des microorganismes qui étaient aussi présents durant la maturation de la choucroute, soit *Kazachstania servazzii* et *Pichia anomala*. Trois souches isolées en usine ont ensuite été évaluées relativement à leur capacité à former un biofilm en laboratoire à l'aide de réacteurs CDC. Les essais en réacteurs CDC ont été réalisés sur du jus de choux en présence du ferment BLAC I ainsi qu'en fermentation spontanée. Des coupons en acier inoxydable étaient utilisés dans les réacteurs CDC et placés à différentes hauteurs. Ceci a permis d'observer une préférence spatiale pour l'interface air-liquide au niveau de la production de biofilm fongique. L'observation de ces surfaces en microscopie électronique a permis de déterminer que l'usage du ferment réduisait l'efficacité des levures à produire un biofilm organisé sur des surfaces complètement submergées en comparaison à des fermentations spontanées. Cette différence n'a toutefois pas été observée à l'interface air-liquide.

Les résultats de cette étude seront utilisés par l'industrie pour augmenter la qualité et la durée de vie des produits commerciaux et auront, par le fait même, un impact majeur pour le secteur de l'industrie de la transformation alimentaire en particulier dans le secteur des aliments fermentés.

Mots-clés : Fermentation, Biofilm, Bactéries lactiques, Levures, Ferment

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INTRODUCTION GÉNÉRALE

1.1 Généralités

La fermentation est utilisée depuis des millénaires pour la production de bière, de vin et de pain, mais surtout pour la conservation des aliments (McGovern et al., 2004). Par contre, le principe derrière ces procédés était autrefois inconnu de ceux qui l'utilisaient. Ce n'est que depuis les recherches de Pasteur en 1857, ayant découvert que les bactéries sont à la source des procédés de fermentation, qu'un intérêt a été développé pour l'étude et la compréhension des principes microbiologiques à la base de cette technique commune (Roissart et Luquet, 1994; Deak, 2007). La fermentation étant traditionnellement utilisée comme méthode de conservation, des procédés complexes ont toutefois été développés pour produire des aliments possédant diverses caractéristiques hautement appréciées des consommateurs. La fermentation a donc été grandement étudiée pour les produits laitiers (yogourt et fromage) et les boissons alcoolisées (bière et vin), mais très peu au niveau des légumes (Aponte et al., 2012). Ceci est principalement explicable en raison de l'efficacité et de la simplicité des méthodes de conservation classiques au niveau des légumes tel que le traitement thermique, l'acidification, le salage, la déshydratation et la réfrigération, sans oublier que la fermentation spontanée est généralement efficace, peu coûteuse et accessible à tous (Deak, 2007).

Il existe aujourd'hui plusieurs légumes fermentés très populaires et ce dans divers pays; le kimchi en Corée, les cornichons aux États-Unis, le miso au Japon et la choucroute en Allemagne qui à elle seule a mené à des revenus d'environ 100 millions d'Euros en 2011 (Medina-Pradas et al., 2017). D'ailleurs, la fermentation convient aux tendances présentement observées dans les habitudes alimentaires des consommateurs, ceux-ci désirant avoir accès à des aliments santé sans agents de conservation et peu ou pas transformés

(Beganović et al., 2014). La fermentation est, de ce point de vue, l'une des meilleures méthodes de conservation puisqu'il s'agit du seul procédé permettant d'améliorer les qualités nutritionnelles et fonctionnelles des aliments en plus d'en assurer la salubrité (Battcock et Azam-Ali, 1998; Breidt, 2005). En effet, le métabolisme des bactéries lactiques fermentaires permet la production de composés naturels connus pour leurs effets bénéfiques sur la santé du consommateur. À titre d'exemple, plusieurs molécules bioactives produites lors de la dégradation des glucosinolates (composés soufrés du chou) sont anticancérigènes, principalement les isothiocyanates (ITC) qui sont produits exclusivement par la fermentation. Certains de ces ITC induisent des enzymes de phase II protégeant contre la toxicité de plusieurs produits chimiques (Tolonen et al., 2002; Peñas et al., 2015). Le chou est aussi reconnu pour ses propriétés antioxydantes (Kusznierewicz et al., 2008; Peñas et al., 2015). De plus, la fermentation nécessite très peu d'équipements, le procédé est économique car il ne nécessite ni chauffage, ni réfrigération et les aliments peuvent être conservés sur une longue période de temps (supérieure à un an), ce qui contribue à l'importance mondiale de cette méthode de conservation (Stamer, 1988; Battcock et Azam-Ali., 1998). C'est d'ailleurs sa passion pour la choucroute qui a permis au célèbre marin britannique, le Capitaine Cook, et à ses équipages, de résister au scorbut (Kodicek et Young, 1969).

1.2 Métabolisme

Lorsqu'on parle de fermentation, il est nécessaire d'aborder le métabolisme des bactéries lactiques. La fermentation est définie comme étant la dégradation de composés organiques par des enzymes provenant de microorganismes en absence d'oxygène. Contrairement à la respiration oxydative, la fermentation mène à l'oxydation partielle des sucres puisqu'elle ne comporte pas de chaîne de transport des électrons et est faite de manière anaérobique (Black, 2008). En l'absence de ce mécanisme, il est nécessaire de transférer l'électron du NAD réduit vers une molécule organique finale pour régénérer le NAD+ nécessaire au début du processus (Black, 2008). Aussi, la fermentation peut procéder sur divers composés organiques comme les acides organiques ou les acides aminés, mais généralement sur les

sucres (glucose, fructose et sucrose dans le chou), et produit peu d'énergie comparativement à la respiration oxydative.

La production de choucroute repose sur deux principaux types de fermentations provenant des principales espèces de bactéries lactiques qui se développent durant le procédé. D'abord, les bactéries du genre *Leuconostoc*, coques à Gram positif retrouvées dans tous les types de sols, vont entamer une hétérofermentation selon la voie des pentoses phosphocetolases qui va mener à la production de multiples composés (CO_2, éthanol, lactate, acétate, mannitol) selon les conditions (Fig. 1.1). En général, la voie hétérofermentaire utilisera deux molécules de glucose pour produire une molécule de lactate, une molécule de gaz carbonique et une molécule d'acétate ou d'éthanol (Roissart et Luquet, 1994; Wisselink et al., 2002). Il y aura donc production d'acides organiques qui possèdent un rôle antimicrobien proéminent en plus d'acidifier le milieu. Ce mécanisme agit envers l'entièreté de la flore indigène du chou, bien que les bactéries lactiques possèdent une résistance accrue aux acides organiques. Comme produit secondaire, cette bactérie produira entre autres du gaz carbonique qui contribuera à la création d'un milieu anaérobique, puis il y aura libération de composés aromatiques qui permettront de développer les arômes du produit final. Ensuite le fructose peut être transformé en mannitol lorsque le fructose agit comme accepteur final d'électrons plutôt que d'être utilisé comme apport en carbone, ce qui contribuera à diminuer la quantité de sucres facilement assimilables dans le milieu (Rodríguez et al., 2012).

Les bactéries du genre *Lactobacillus* sont des bâtonnets à Gram positif, non sporulantes et sont généralement retrouvées dans le système gastro-intestinal de l'humain et des animaux. Leur métabolisme est anaérobie facultatif mais elles se comportent comme des homofermentaires en présence d'hexoses. L'homofermentation, voie métabolique utilisée par *Lactobacillus plantarum* en présence d'hexoses, est beaucoup plus prévisible. En effet, ce microorganisme génère la production exclusive de lactate à partir de glucose selon la voie d'Embden-Meyerhof-Pranas, ce qui mène à la plus grande part de l'acidification du milieu (Fig. 1.2).

Ceci étant aussi possible en raison de sa résistance accrue à la baisse du pH intracellulaire via des pompes à protons très efficaces (McDonald et al., 1990; Roissart et Luquet, 1994).

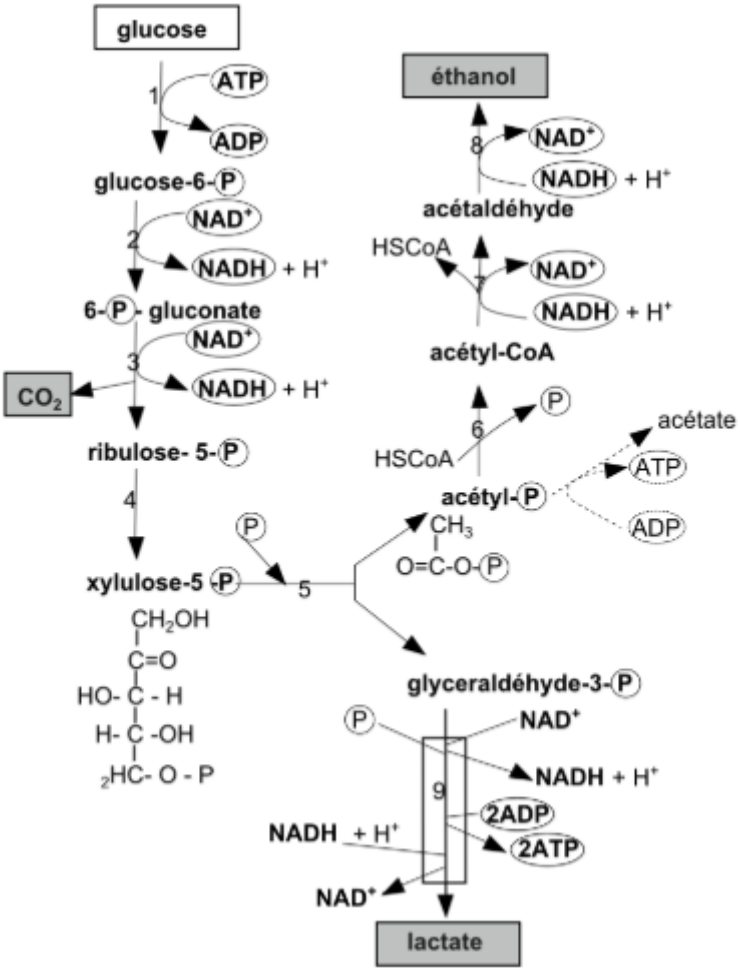

Figure 1.1 : Schéma métabolique de la voie des phosphocétolases (hétérofermentaire). Reproduit avec la permission de JF Perrin.

Figure 1.2 : Schéma métabolique simplifié de la voie de Embden-Meyerhof-Pranas (homofermentaire). Reproduit avec la permission de JF Perrin.

Bien que l'on puisse penser que la production d'acétate possède un rôle mineur dans la stabilité du produit final en terme d'acidification, il en est tout autre pour son activité antimicrobienne. Effectivement, l'acide acétique ayant un pKa plus élevé que l'acide lactique, cette molécule se retrouvera donc majoritairement sous forme indissociée dans le milieu. Ceci, combiné à son faible poids moléculaire, lui permet de pénétrer passivement la membrane des cellules bactériennes afin de se dissocier dans le cytoplasme et ainsi acidifier le milieu intracellulaire des microorganismes. L'acétate annule donc le potentiel membranaire des cellules, ce à quoi une faible diversité de microorganismes sont aptes à survivre (Wiander et Ryhänen, 2005).

Un autre genre a aussi été associé à la production de choucroute dans la littérature scientifique, soit *Pediococcus acidilactici* (Etchells et al., 1973; Medina-Pradas et al., 2017). Cette espèce est retrouvée sous forme de coques à Gram positif et possède un métabolisme fermentaire homolactique, tel que celui de *Lactobacillus plantarum*. *Pc. acidilactici* peut produire différentes bactériocines ayant un effet antimicrobien sur un large éventail de bactéries (Bhunia et al., 1988; Biswas et al., 1991). Cette bactérie intervient majoritairement dans des fermentations s'opérant à températures élevées comme dans le cas des olives (Medina-Pradas et al., 2017).

1.3 Légumes biologiques versus conventionnels

Depuis plusieurs années, une augmentation accrue de la consommation d'aliments biologiques est observée partout dans le monde. Seulement aux États-Unis, on parle d'une augmentation de 40 fois entre 1986 et 1996 (Bourn et Prescott, 2002). Lorsque vient le temps de comparer les deux modes de production, de nombreux facteurs doivent être pris en compte; économie, productivité, qualité, environnement et bien d'autres. Toutefois, les facteurs prédominants sont ceux de la qualité et de la salubrité des légumes. Ainsi, trois principaux aspects sont relevés par Bourn et Prescott (2002) pour comparer le biologique au conventionnel; la valeur nutritionnelle, la qualité sensorielle et la sécurité alimentaire. Tout d'abord, la valeur nutritionnelle a été le sujet de nombreuses études portant sur l'analyse chimique, l'analyse au niveau de la ferme ferme, l'effet de fertilisants et l'effet sur la santé (El Gindy et al., 1957; Lieblein, 1993; Abell et al., 1994). Malgré le nombre d'études portant sur le sujet, plusieurs raisons expliquent qu'aucun consensus ne soit encore atteint à ce jour. La disparité des études, le manque de rigueur statistique et la grande quantité de variables en sont les principales. Toutefois, certaines études de qualité ont permis de déterminer que le cultivar avait un rôle prédominant comparé au sol et au climat (El Gindy et al., 1957). Aussi, malgré la grande variabilité des résultats, moins de nitrates sont observés pour certains légumes biologiques (observé en autres pour le chou) alors qu'aucune différence significative n'est décrite pour les minéraux, vitamines, sucres ou autres composés (Stopes et al., 1988; Lieblein, 1993; Warman et Havard, 1998; Magkos et al., 2003).

Au niveau de la qualité sensorielle, contrairement aux croyances populaires, les légumes biologiques n'ont pas démontré d'avantages par rapport aux légumes conventionnels en raison de la complexité de telles études. Les principaux facteurs semblant influencer la qualité sensorielle sont les caractéristiques du sol, le climat et le temps de récolte du légume, soit des facteurs variant d'un champs à l'autre, peu importe le type de culture y étant cultivé

(Bourn et Prescott, 2002). Une autre étude sur la qualité visuelle en est arrivée aux mêmes conclusions (Conklin et Thompson, 1993).

Pour ce qui est de la sécurité alimentaire, quelques études ont pu démontrer l'absence de résidus chimiques sur les fruits et légumes biologiques comparativement aux conventionnels qui en contenaient davantage (Vannoort et al., 1997; Magkos et al., 2003). Cependant, les niveaux détectés étaient toujours inférieurs aux limites tolérées (Bourn et Prescott, 2002). Au niveau microbiologique, il ne semble pas y avoir de risque accru pour les cultures biologiques. Bien qu'un nombre croissant d'intoxications alimentaires reliées à la consommation de légumes crus soit recensé, aucune étude n'a pu démontrer que ceci pourrait être relié au type de culture utilisé, mais il est possible de penser que la flore autochtone soit différente (Magkos et al., 2003). C'est pourquoi nous avons voulu évaluer ce facteur.

1.4 Procédé de fermentation du chou

1.4.1 Prétraitement

Le procédé utilisé dans la production industrielle de choucroute se déroule généralement en quatre grandes phases, résumées dans le tableau 1.1. Bien qu'on identifie diverses phases, il importe de se rappeler qu'il s'agit d'un processus séquentiel mixte. Initialement, lors de la phase appelée prétraitement, le chou est coupé et le cœur ainsi que les feuilles externes (généralement sales ou jaunies) sont retirés. Le chou est ensuite taillé en fines lanières (environ 10 x 50 mm), puis mélangé avec du sel ou une saumure. Ceci permet de créer un milieu de culture propice à la croissance des bactéries lactiques. Le salage et le coupage mènent à la plasmolyse des cellules végétales, ce qui aide à l'extraction graduelle des sucs tissulaires (principalement glucose et fructose) via osmose, ce qui permet au milieu de soutenir

la croissance microbienne. Le légume est ensuite chambré dans un récipient et pressé afin de promouvoir à nouveau la lyse cellulaire en plus d'éliminer les poches de gaz éventuelles. Les récipients sont alors scellés de manière à empêcher les échanges gazeux avec l'air ambiant tout en permettant la sortie du gaz carbonique produit durant la fermentation. Ainsi, lors du prétraitement, il y a formation d'un milieu anaérobique avec faible potentiel redox par la consommation de l'oxygène résiduel par les microorganismes aérobies ainsi que par les cellules végétales. La fermentation se déroule ensuite à une température d'environ 20 ^{o}C dans le cas du chou, alors que les olives se fermentent généralement à des températures plus élevées et, à l'opposé, le kimchi se fermente à des températures proches de 10 ^{o}C. Cependant, les fermentations à grande échelle sont généralement faites sans contrôle précis de la température.

Tableau 1.1 : Résumé des phases de fermentation du chou.

	Phase I	Phase II	Phase III	Phase IV
Microorganisme principal	Flore indigene	*Lc. Mesenteroides*	*Lb. Plantarum* *Pc. acidilactici* *Lb. brevis*	-
Type de métabolisme	Aérobique	Hétérofermentation	Homofermentation	Maturation, Aucune croissance
Produits principaux	-	CO_2, Acétate, Lactate, Mannitol	Lactate	-
Changements du milieu	Milieu anaérobique Augmentation des sucres libres	Acidification initiale à un pH de 4,00	Acidification finale à un pH de 3,30-3,50	Stabilisation chimique, estérification

1.4.2 Hétérofermentation

La fermentation lactique du chou se caractérise ensuite par la phase d'hétérofermentation qui marque l'acidification initiale du milieu jusqu'à un pH d'environ 4,0. Cette étape dure généralement de 3 à 5 jours, variant selon l'inoculum initial en bactéries lactiques, l'usage d'un ferment ou non, la température et le volume de la fermentation tel que rapporté par Savard et al., (2000). La vitesse d'initiation de l'hétérofermentation est cruciale dans le procédé puisqu'un délai prolongé entre les phases I et II (Tableau 1.1) permet aux bactéries pathogènes de produire des toxines et les microorganismes néfastes peuvent altérer le produit, ce qui joue grandement sur les qualités organoleptiques du produit final (Viander et al., 2003). Certains microorganismes peuvent aussi s'adapter à l'acidification, c'est le cas entre autres de certaines levures qui développent une acido-résistance relative. Le microorganisme principal de la phase hétérofermentaire est *Leuconostoc mesenteroides*. La phase d'hétérofermentation est particulièrement importante parce que la production des acides lactique et acétique inhibe rapidement la flore commensale du chou qui est incapable de s'adapter à l'acidification rapide du milieu. La production de gaz carbonique permet de créer un environnement anaérobique, puis la bioconversion du fructose en mannitol nuit à la croissance des levures (Rodríguez et al., 2012). À la fin de cette phase, les bactéries du genre *Leuconostoc* entrent en phase stationnaire en raison de leur autoinhibition face à la production d'acides organiques.

1.4.3 Homofermentation

Apparaît ensuite, mais presque de façon simultanée, la phase d'homofermentation qui mènera à l'acidification finale du milieu. Cette étape débute lorsque les bactéries du genre *Lactobacillus* prennent le dessus sur *Ln. mesenteroides*. *Lb. plantarum* utilise la voie d'Embden-Meyerhof-Parnas afin de produire uniquement du lactate lors de la fermentation en présence d'hexoses (Wisselink et al., 2002; Breidt, 2005). Ceci mène à la disparition de *Ln. mesenteroides* puisque cette bactérie ne peut supporter une aussi grande diminution de son

pH intracellulaire comparativement à *Lb. plantarum* qui continue à produire exclusivement de l'acide lactique en grande quantité (McDonald et al., 1990). La production de lactate (jusqu'à 2%) accentue donc grandement l'acidification du milieu, permettant d'atteindre un pH final entre 3,3 et 3,5, variant selon les souches présentes. Il est aussi connu que *Lb. brevis* est capable de terminer la fermentation initiée par *Lb. plantarum* en consommant une partie des sucres résiduels suite à l'inhibition de cette dernière par l'acide lactique et ce en raison de la plus grande résistance de *Lb. brevis* aux pH acides (Medina-Pradas et al., 2017). Toutefois, cette dernière n'est pas toujours présente dans le procédé.

1.4.4 Maturation et fermentation secondaire

Finalement, débute ce qu'on appelle la phase de maturation lorsque l'activité microbienne s'arrête. Au cours de cette étape, qui peut s'étaler sur plusieurs mois, il ne devrait y avoir aucune croissance bactérienne, mais plutôt une stabilisation chimique. Durant ce stade, il y a une certaine activité enzymatique qui permet le peaufinage des arômes et autres qualités organoleptiques de la choucroute. Cependant, il est possible d'y observer la croissance de levures acido-résistantes si tous les sucres fermentescibles n'ont pas été métabolisés. Ces dernières sont indésirables de par leur capacité à produire du gaz et de la capacité de certaines espèces à consommer les acides organiques comme source de carbone en présence d'oxygène (Franco et Pérez-Díaz, 2012). Le mode de fermentation le plus répandu pour les levures est la fermentation alcoolique. Dans cette voie métabolique, un acide pyruvique est transformé en une molécule de CO_2 et d'acétaldéhyde, puis cette dernière est métabolisée en alcool éthylique (Black, 2008). Plusieurs facteurs influencent la disponibilité des sucres résiduels après la fermentation lactique, soit la concentration initiale en sucres, le pH, la concentration en sel, la température et le pouvoir tampon du légume (Fleming et al., 1985; Medina-Pradas et al., 2017). Selon ces facteurs, les bactéries lactiques peuvent en arriver à leur autoinhibition par la concentration d'acides organiques avant d'avoir métabolisé la totalité des sucres

fermentescibles ce qui représente un risque qui est généralement contrôlé par l'utilisation de ferments.

1.5 Facteurs influençant la qualité des fermentations de légumes

Naturellement, la microflore indigène du chou est composée de 0,15 à 1,5% de bactéries lactiques seulement (Buckenhüskes et al., 1997). Suite au prétraitement, une fermentation spontanée peut se produire lorsque l'aliment est placé dans des conditions favorisant la croissance des rares bactéries lactiques plutôt que la croissance des levures, moisissures et bactéries commensales et pathogènes présentes en bien plus grandes proportions (Buckenhüskes et al., 1997). Étant donné la grande variabilité présente dans cette microflore causée par le climat, le type de sol, les engrais et les pesticides, une fermentation spontanée basée sur cette faible proportion de bactéries lactiques peut difficilement être contrôlée adéquatement et de manière reproductible d'année en année (Gardner et al., 2001; Medina-Pradas et al., 2017).

La qualité d'une fermentation de chou est donc basée sur le pH final, les niveaux et le ratio d'acides organiques, les qualités organoleptiques et la reproductibilité du résultat final. Selon Roissart et Luquet (1994), un ratio entre 3:1 et 5:1 de lactate:acétate est indispensable pour obtenir une choucroute de bonne qualité. Bien qu'il soit relativement facile de débuter une fermentation spontanée de légumes, certaines lignes directrices ont été développées afin d'obtenir une fermentation optimale et standardisée.

1.5.1 Disponibilité des sucres et température

La concentration des substrats et leur disponibilité hors des tissus végétaux sont nécessaires pour soutenir la croissance microbienne et permettre la production des acides organiques par les bactéries lactiques. Le prétraitement, le type de légume et le salage auront un impact sur la quantité de sucres disponibles pendant et après la croissance des bactéries lactiques. Lors du prétraitement du légume, le fait de couper le chou avant la fermentation permet de rendre les sucres disponibles plus rapidement pour les bactéries lactiques. Le sel contribue ensuite à la lyse des cellules végétales, ce qui permet d'en extraire les sucres, et selon le type de légume ou le cultivar la concentration en sucre sera variable, donc par le fait même la quantité totale de sucres disponibles pour la fermentation.

La température à laquelle se déroule la fermentation influencera la durée des différentes phases de fermentation mais aussi la séquence bactérienne. Il a été démontré qu'une choucroute fermentée à plus basse température (18 ^{o}C) serait de qualité supérieure au niveau de sa couleur, son goût et sa fermeté comparativement à une fermentation à haute température (32-37 ^{o}C) puisque les bactéries lactiques ont une croissance plus lente à température élevée comparativement aux bactéries pathogènes et celles qui dégradent le légume (Medina-Pradas et al., 2017). Aussi, ces conditions écourtent la période de dominance de *Ln. mesenteroides* en raison de la croissance plus rapide de *Lb. plantarum*, ce qui modifie grandement la durée des phases d'homo- et d'hétérofermentation et donc le ratio final en acides organiques ainsi que la concentration des différents composés secondaires aromatiques produits lors de la phase hétérofermentaire.

1.5.2 Taux de sel

La concentration optimale de chlorure de sodium se situe entre 2 et 3% pour la choucroute, ce qui favorise la croissance des bactéries lactiques et contribue à conserver la fermeté du chou en empêchant la dégradation des pectines en plus, bien sûr, de favoriser la plasmolyse des cellules végétales (Pederson et Albury, 1969; Medina-Pradas et al., 2017). Comme la production de choucroute crée beaucoup d'eaux usées en raison de la quantité de sel menant à un excès de jus de choucroute dans le produit final, plusieurs études ont été faites afin de réduire la concentration en sel dans le procédé (Hang et al., 1972). Ainsi, l'utilisation de ferments a permis à plusieurs reprises d'obtenir des fermentations stables en réduisant significativement le taux de sel nécessaire, ce qui mène ultimement à une réduction de la quantité d'eau extraite des feuilles de chou et de la quantité de sel utilisé et d'eaux usées à traiter (Breidt, 2005; Johanningsmeier et al., 2007; Beganović et al., 2011). Cette amélioration permet aussi de réduire la quantité de sel dans le produit final, ce qui est positif pour la santé des consommateurs. Cela contribue donc à l'appellation d'aliment santé qui caractérise la choucroute (Viander et al., 2003). Cependant, une réduction accrue en sel dans le procédé peut faciliter la croissance des bactériophages et ainsi nuire aux bactéries lactiques tout en favorisant la survie des bactéries pathogènes (Mudgal et al., 2006). C'est pourquoi le taux de sel n'est pas une variable que l'on peut modifier à la légère.

1.5.3 Usage d'un ferment

Plusieurs facteurs peuvent influencer la qualité du produit final, mais le principal est la microflore et donc l'usage d'un ferment permet d'initier la fermentation plus rapidement et ainsi d'accélérer la production d'acides organiques en début de fermentation et il permet aussi d'obtenir une plus grande acidification finale. Le taux de croissance des microorganismes en

début de fermentation permet de contrôler la croissance de microorganismes nuisibles rapidement dans le processus (Fleming et al., 1985; Medina-Pradas et al., 2017).

Il existe trois principaux procédés de fermentation dans le cas des aliments en général: la fermentation spontanée, la fermentation avec ajout d'un ferment de culture pure et la fermentation avec ajout d'un ferment mixte. Dans le cas des légumes, la fermentation spontanée est la principale méthode utilisée mondialement, puisqu'il s'agit de la technique la plus simple et qu'elle fonctionne assez bien, mais nécessite toutefois une pasteurisation. Une telle fermentation permet l'obtention d'un goût typique local en raison de la flore indigène du chou qui varie selon les régions en raison du climat et du sol. Cependant, à cause de cette variation, la fermentation spontanée mène à une faible standardisation au niveau de la qualité du produit final d'année en année. De plus, la pasteurisation diminue grandement les qualités nutritionnelles et organoleptiques de l'aliment.

Il est aussi possible d'utiliser une production antérieure afin d'inoculer une nouvelle fermentation comme cela existe pour les produits laitiers. Par contre, cette méthode, appelée «technique du pied de cuve», amène encore certaines irrégularités puisque l'inoculum et les concentrations des microorganismes seront différents selon le stade durant lequel le prélèvement est fait pour inoculer la nouvelle production (Xiong et al., 2014). Ainsi, cette technique est similaire à la fermentation spontanée, mais permet une initiation un peu plus rapide du processus en raison de la plus grande concentration en bactéries lactiques comparativement à la microflore naturelle du chou.

1.5.3.1 Ferments de culture pure

Plusieurs études ont été faites pour tenter de produire des ferments de culture pure qui seraient ajoutés à la microflore indigène, mais en proportion beaucoup plus grande que dans les autres genres d'aliments. Dans le cas des légumes, le défaut de cette méthode est qu'elle ne tient pas compte du fait que la fermentation du chou est un procédé d'interactions microbiologiques complexe nécessitant différents types de bactéries lactiques tel que décrit dans la section 1.2. Ainsi, inoculer avec une culture pure de *Ln. mesenteroides* permet d'initier la fermentation rapidement, mais la phase de fermentation lactique (homofermentation) est moins efficace ou même concurrente et le produit risque de comporter un trop grand taux d'acide acétique, menant à un goût vinaigré accentué (Xiong et al., 2014). Inversement, une inoculation avec *Lb. plantarum* cause un manque d'acide acétique pour la quantité d'acide lactique produite, limitant l'action antimicrobienne (Xiong et al., 2014). Le ratio des acides organiques étant important dans la production de choucroute, la fermentation spontanée permet d'obtenir un produit préférable pour le consommateur et de qualité supérieure à l'usage de ferments simples puisque cette méthode nuit au processus séquentiel mixte observable lors de la fermentation du chou (Andersson et al., 1990).

1.5.3.2 Ferments de culture mixte

En comparaison avec la fermentation spontanée ou à l'utilisation d'un ferment à une souche, un ferment mixte permet une acidification plus rapide et standardisée d'une production à l'autre tout en reproduisant parfaitement le déroulement normal d'une fermentation spontanée mais améliorée. Ceci en raison de la sélection de souches biocompatibles avec un haut potentiel fermentaire. L'usage de différents types de microorganismes apporte un contrôle plus complet, soit sur chaque phase de la fermentation ce qui permet d'obtenir un bon ratio lactate:acétate.

Ainsi, un ferment mixte permet d'obtenir des qualités organoleptiques recherchées par les consommateurs avec un produit final standardisé (Potts et Fleming, 1982; Font de Valdez et al., 1990). Cette efficacité mène directement à une diminution des pertes reliées à l'initiation lente des fermentations spontanées ainsi qu'une réduction des risques de fermentation secondaire dû à un métabolisme plus complet des sucres (croissance de microorganismes nuisibles, dégradation du chou, oxydation et brunissement du chou en surface). Une augmentation de la durée de vie du produit est aussi constatée en raison de l'acidification plus rapide et d'une meilleure utilisation des sucres et, pour principal avantage, ne nécessite pas de pasteurisation.

Par contre, ce type de fermentation comporte aussi certains inconvénients comme le risque de développement de bactériophages spécifiques aux souches du ferment, le risque de perte de plasmides ou caractéristiques métaboliques du ferment, le coût et les équipements nécessaires à la production de ce dernier. En résumé, l'usage d'un ferment mixte est préférable à un ferment de culture pure puisqu'il est beaucoup plus représentatif du procédé naturel donc spontané et permet un meilleur contrôle de chacune des étapes de la fermentation. De plus, l'usage d'un ferment mixte permet d'obtenir une fermentation de qualité supérieure à la fermentation spontanée sans nécessiter de pasteurisation ou ajout d'agents chimiques de conservation comme les benzoates et sorbates (Gardner et al., 2001; Johanningsmeier et al., 2007).

1.5.3.3 Développement d'un ferment et ferment BLAC I

Lors du développement d'un ferment, il faut tenir compte de plusieurs caractéristiques des bactéries pour obtenir un produit final de bonne qualité. Ainsi, l'activité acidifiante, l'activité arômatisante et l'activité inhibitrice des souches envers la microflore indigène du chou sont à considérer. La croissance de *Ln. mesenteroides* est donc très importante en début de

fermentation pour sa production d'acide acétique menant à une perception d'acidité agréable pour le consommateur lorsqu'en bonne proportion. Cet acide permettrait aussi aux feuilles de chou de rester plus croquantes en protégeant contre le ramollissement causé par l'acide lactique (Fleming et al., 1985). La capacité des bactéries à utiliser la totalité des sucres fermentescibles du milieu est aussi très importante pour réduire les risques de fermentation secondaire des sucres résiduels par les levures acido-résistantes. Dans le cas où peu de sucres résiduels sont présents, une pasteurisation ou autre traitement thermique ne sont pas nécessaires, ce qui évite de réduire les qualités nutritionnelles de l'aliment.

Basé sur ces critères, un ferment mixte pour légumes a été développé et a fait son entrée sur le marché industriel en 1997 dans le but de standardiser la production de légumes fermentés, mais principalement la choucroute. Ce ferment, nommé BLAC I, contient des souches sélectionnées de *Ln. mesenteroides*, *Lb. plantarum* et *Pc. acidilactici* et a été développé par l'équipe du Dr Savard (Gardner et al., 2001). L'utilisation de ce ferment permettait d'obtenir un produit stable sans pasteurisation et inhibant efficacement la croissance de levures. Aussi, le ferment a été démontré plus efficace et donnant un produit de qualité supérieure face à une fermentation spontanée par son acidification élevée et sa durée de vie accrue (Gardner et al., 2001). Ici, l'usage de *Pc. acidilactici* permet, entre autres, de contrôler les fermentations en cas de températures supérieures à 20 ^{o}C et ainsi éviter le développement rapide de bactéries pathogènes et la production de toxines dans le légume en début de fermentation. Ce ferment est utilisé depuis plus de 20 ans et ses qualités ont été remises en doute dans les dernières années, principalement en lien avec la réapparition de levures acido-résistantes observée en industrie et dans nos laboratoires. Les présents travaux constituent donc une investigation sur les pistes pouvant causer la recrudescence de ces levures.

1.6 Conservation et dégradation de la choucroute

Les aliments fermentés sont composés d'une multitude de barrières contre la croissance de bactéries pathogènes comme les acides organiques, le sel et les bactériocines, mais aussi le pH, l'anaérobiose, le potentiel redox bas et le peroxyde d'hydrogène et e diacétyle. Les bactériocines sont décrites comme étant des molécules inhibant la croissance d'espèces proches de celle les produisant. De plus, quelques bactéries lactiques produisent aussi des lantibiotiques qui ont un spectre beaucoup plus large, pouvant ainsi s'attaquer à des bactéries pathogènes. Ceci démontre bien la relation d'amensalisme qui se produit dans la fermentation du chou plutôt qu'une relation de mutualisme ou de synergisme généralement observable dans les aliments fermentés tel que le yogourt (Ivey et al., 2013).

Bien que les aliments fermentés comportent une importante combinaison de barrières pour le contrôle de la croissance des microorganismes nuisibles, certaines levures acido-résistantes peuvent survivre à la fermentation et dégrader la choucroute durant ou après la maturation (Moon, 1983). La fermentation secondaire survient lorsqu'il y a croissance fongique dans les cuves de fermentation contaminées par des levures résistantes aux acides organiques ou suivant le processus d'emballage. Ici, la phase d'hétérofermentation joue un rôle très important, puisque la transformation du fructose en mannitol permet de réduire grandement les sucres assimilables par les levures (Rodríguez et al., 2012). En effet, le mannitol est un sucre-alcool que les levures sont incapables de métaboliser par fermentation à pH acide (Barnett, 1976). Les levures peuvent donc métaboliser uniquement le glucose et le fructose n'ayant pas été utilisé par les bactéries lactiques avant leur auto-inhibition par la production d'acides organiques. Au-delà de nuire aux qualités organoleptiques, la fermentation des levures peut causer des risques importants au niveau sanitaire. En présence d'oxygène, certaines levures sont capables de dégrader le lactate et l'acétate, ce qui mène à une augmentation du pH et ainsi à une diminution des barrières de contrôle des agents pathogènes (Franco et Pérez-Díaz, 2012). Une autre importante cause de pertes industrielles reliée à la croissance des levures est

le gonflement des emballages causé par la production de gaz suite à l'incorporation d'oxygène involontaire durant la mise en sachets ou en pots (Moon, 1983; Thomas et Davenport, 1985). L'origine de ces levures pouvant expliquer leur présence en fin de fermentation, est donc l'objet de la présente étude.

1.7 Biofilms dans l'industrie alimentaire

Même si l'utilisation de ferments permet de contrôler les écarts de fermentation, l'utilisation répétée des cuves de fermentation peut mener au développement de biofilms. Les biofilms sont décrits comme étant un assemblage de polymères extracellulaires produits par des bactéries ou levures attachées à une surface qui trappe des microorganismes à l'intérieur de cette matrice organique (Sauer et al., 2007). Cette adhérence contribue grandement à la persistance des microorganismes et leur résistance au nettoyage mécanique dans un environnement riche en nutriments ou encore, dans le cas qui nous intéresse, leur résistance aux acides organiques. Au-delà de la simple adhésion, il s'agit d'une matrice protectrice généralement très efficace contre les désinfectants et composés antimicrobiens de tout genre. Le biofilm permet le passage d'eau et de nutriments mais peut aussi réduire la pénétration des assainissants et autres composés inhibant la croissance des microorganismes (Shi et Zhu, 2009).

La formation de biofilms est un problème majeur rencontré dans l'ensemble de l'industrie de la transformation alimentaire. Une grande proportion de microorganismes semblent avoir la capacité de former un biofilm pour s'ancrer sur les surfaces, qu'elles soient en bois, verre, plastique, polystyrène, acier inoxydable ou autres (Arnold et Bailey, 2000; Didienne et al., 2012). Bien que la découverte des biofilms remonte à près d'un siècle (Zobell, 1943), il s'agit toujours d'un problème d'actualité en transformation de la viande, production de bière, d'aliments fermentés ainsi que dans l'ensemble de l'industrie alimentaire

(Timke et al., 2008; Cloete et al., 2009; Srey et al., 2013). Au fil du temps, de nombreuses méthodes ont été développées pour réduire les impacts de cette persistance microbienne. Ainsi, le simple nettoyage et la désinfection ont été combinés à l'utilisation d'enzymes, de bactériophages, d'autres métabolites et même l'utilisation de bactéries lactiques pour réduire les risques de contaminations reliées aux biofilms (Simões et al., 2010). Les biofilms sont particulièrement dangereux dans l'industrie des viandes et de la volaille où il peut y avoir formation de foyers de bactéries pathogènes. Dans les autres cas, il est généralement question de microorganismes causant la détérioration du produit bien que la transformation de produits végétaux ait aussi été le siège d'épidémies sans jamais toutefois avoir affecté les légume fermentés (Shi et Zhu, 2009).

Dans le cas de la production industrielle de choucroute, il est possible que des levures survivent grâce à la présence de ces biofilms. Celles-ci pourraient ensuite contaminer le produit entre l'ouverture des cuves et l'emballage. L'introduction d'oxygène durant le processus contribuerait à une croissance plus rapide des levures néfastes, pouvant ainsi utiliser plus de substrats (Franco et Pérez-Díaz, 2012).

1.8 Problématique et buts de l'étude

Au cours des dernières années, une augmentation de la variabilité des produits fermentés avec le ferment BLAC I a été observée par une plus grande survie des levures et une mortalité accrue du ferment lors de la phase de maturation. Plusieurs hypothèses ont été avancées pour expliquer cette variabilité. La principale étant l'évolution normale de la microflore indigène du chou devenant plus compétitive face au ferment. Aussi, le ferment était autrefois plus efficace au niveau de la fermentation du chou biologique, alors qu'il semble y avoir une inversion de

ce phénomène. Le chou conventionnel offrirait un produit plus stable lorsque fermenté avec le ferment BLAC I. Ceci a mené à la première partie du projet qui consistait en l'étude de l'écologie microbienne des fermentations de légumes conventionnels et biologiques.

Dans cette section, une production de choucroute à l'échelle industrielle a été investiguée au niveau de l'écologie microbienne et de l'efficacité des fermentations en comparant l'usage de chou conventionnel à du chou biologique provenant d'un même producteur et concernant le même cultivar. Puis, l'utilisation d'un ferment versus une fermentation spontanée a aussi été comparée. Le tout suivi d'une identification aléatoire des souches isolées lors des dénombrements microbiens.

D'autre part, l'apparition de levures acido-résistantes responsables de fermentations secondaires ayant posé un nouveau problème à l'industrie, l'hypothèse que des biofilms fongiques pouvaient être à l'origine du problème a alors été avancée. La deuxième partie du projet consistait donc à étudier l'impact des levures acido-résistantes et le développement potentiel de biofilms dans des cuves industrielles en acier inoxydable. L'existence des biofilms fongiques à la surface interne des cuves de fermentation au niveau de l'interface air-liquide n'a toutefois, à notre connaissance, jamais été démontrée. Bien que les cuves soient refermées avec une double pellicule de plastique contenant de l'eau chlorée afin d'assurer l'anaérobiose du milieu, il est fort probable que des poches d'air soient créées en raison des plis formés dans le plastique, limitant ainsi l'effet inhibiteur des acides organiques dans certaines zones de la couverture ce qui pourrait contribuer à la formation de biofilms et de microenvironnements. La capacité des principales levures de contamination à former des biofilms fongiques a été évaluée en laboratoire sous diverses conditions afin de déterminer la capacité des bactéries lactiques à inhiber cette contamination.

Somme toute, ce projet de recherche avait pour but de (1) documenter l'écologie microbienne des fermentations lactiques des légumes (2) vérifier la présence de levures acido-résistantes dans les biofilms potentiels ainsi que d'évaluer leur capacité à produire un biofilm fongique durant la fermentation si de telles levures étaient identifiées.

CHAPITRE 2

ÉTUDE DE L'ÉCOLOGIE MICROBIENNE D'UNE PRODUCTION DE CHOUCROUTE AVEC ET SANS FERMENT ET AVEC DU CHOU BIOLOGIQUE OU CONVENTIONNEL DANS DES CUVES DE FERMENTATIONS INDUSTRIELLES EN ACIER INOXYDABLE

L'écologie microbienne de production de choucroute a été investiguée à l'échelle industrielle et laboratoire sur une durée de 180 jours. Des dénombrements de comptes viables et des analyses biomoléculaires pour l'identification des espèces ont été effectués pour quatre conditions différentes. Des choucroutes ont été produites avec du chou conventionnel et biologique par fermentation spontanée et avec un ferment mixte. Les échantillons ont été prélevés en surface et en bas des cuves industrielles et ont permis l'identification de deux levures récurrentes, *Kazakhstania servazzii* et *Pichia anomala* en surface alors qu'aucune levure n'a été détectée en bas de cuve. De plus, à l'échelle laboratoire, aucune levure n'a pu être détectée. L'observation d'une séparation spatiale des populations fongiques, favorisées en haut de cuve, pourrait signifier que les bactéries lactiques peuvent inhiber partiellement la croissance des levures, ce qui pourrait être amélioré par une modification du procédé de fermentation actuel. Cette étude a confirmé la grande efficacité de l'usage d'un ferment mixte et sa capacité à standardiser le produit même en présence de chou provenant de différents champs. Le mode de production de chou, biologique ou conventionnel, ne semble pas être une source de variation lors du procédé de fermentation avec un ferment. Les seules différences observées entre l'échelle industrielle et laboratoire sont reliées à la croissance de levures et à la séparation spatiale des microorganismes et acides organiques en début de fermentation.

Diverses personnes ont contribué à cette étude. Leurs rôles distinctifs sont : j'ai produit les résultats d'écologie microbienne des fermentations à l'échelle industrielle, ainsi que la totalité

des identifications biomoléculaires et des quantifications de sucres et acides au HPLC avec le support des assistants de recherche. J'ai aussi analysé les résultats et écrit les premières versions de l'article. Caroline Lapointe a effectué les essais d'écologie microbienne à l'échelle laboratoire et a participé à leur analyse. Les Dr Tony Savard et Dr Carole Beaulieu ont contribué à l'analyse des résultats et ont révisé les premières versions de l'article. Le Dr Tony Savard a élaboré les objectifs et le détail du projet.

STUDY OF THE BACTERIAL ECOLOGY OF SAUERKRAUT PRODUCTION WITH AND WITHOUT STARTER CULTURE USING ORGANIC AND CONVENTIONAL CABBAGE IN INDUSTRIAL STAINLESS STEEL FERMENTATION VATS

Yanick Bisaillon [a, b], Caroline Lapointe [a], Carole Beaulieu [b], Tony Savard [a]

[a] *Agriculture and Agri-Food Canada*

[b] *Université de Sherbrooke*

ABSTRACT

The microbial ecology of sauerkraut productions was investigated at industrial and laboratory scales over a 180 day process. Viable counts and identification of the species by biomolecular analysis were performed on four different conditions were carried out. Sauerkraut with conventional and organic cabbage was produced under spontaneous fermentation and with the use of a mixed microbial starter culture. Samples were taken at the top and the bottom of the vats at different time and allowed the identification of two recurring yeast strains, *Kazachstania servazzii and Pichia anomala* exclusively found at the surface, as no yeasts were detected either at the bottom of the vats or from the laboratory scale trials. The observation of a spatial separation in the fungal populations could mean that lactic acid bacteria are able to partially inhibit yeast growth and could be improved with a modification of the fermentation process. This study confirmed the high efficiency of the mixed microbial starter culture and its capacity to standardize the product even with cabbage from different fields. Organic and conventional cabbage did not seem to be a source of variation in the fermentation process with a starter. The only differences observed between industrial and laboratory scale were related to the yeast growth and spatial distribution of the microorganisms.

INTRODUCTION

The preservation of vegetables is generally achieved with the use of different processes like refrigeration, freezing, thermal treatment, acidification or fermentation. However, this last process shows a special interest because of its added value that provides both safety and better nutritional and functional qualities to foods (Breidt, 2005). The presence of salt, the low redox potential, the rapid and strong acidification, the presence of organic acids, the production of hydrogen peroxide and other antimicrobials compounds are building a strong combination of hurdles to ensure the safety of fermented foods (Fleming et al., 1985; Medina-Pradas et al., 2017). Another important hurdle is the presence of bioactive compounds. It has been reported that glucosinolates (a sulfur component naturally present in the cabbage leaves) are completely metabolized into other antimicrobial compounds during the fermentation process (Kyung and Fleming, 1994; Palani et al., 2016). Mostly allyl isothiocyanates (AITC) and methyl methanethiosulfinates (MMTSO) are produced, which have antimicrobial effects. Moreover, it is known that many of the glucosinolates subproducts have anticarcinogenic capacities and that some of them activate anticancer pathways (Kyung and Fleming, 1994; Rhodes, 1996; Kyung and Fleming, 1997; Tolonen et al., 2002).

Over the past decades, an increased augmentation in the consumption of organic foods has been noticed (Bourn and Prescott, 2002). Although certain consumers believe that they are better than conventional in nearly every aspect, it has yet to be scientifically proven. Studies are based on three principal categories; nutritional value, sensory quality and food safety. Of all the research that were done on this subject, very few were well controlled and used good statistical models (Bourn and Prescott, 2002). It was demonstrated that the influence on minerals and proteins is caused principally by the cultivar used, over the ground and climate (El Gindy et al., 1957). Also, the nitrate value (higher in conventional foods) is the only nutritional value that was repeatedly shown different for cabbage (Meier-Ploeger et al., 1989; Lieblein, 1993; Warman and Havard, 1998). No significant sensory qualities differences were

observed (Conklin and Thompson, 1993; Bourn and Prescott, 2002). Similar observations were noted for food safety, as organic foods are not microbiologically at risk, but often contain less chemical residues (Magkos et al., 2003).

The present study focuses on the fermentation of cabbage to produce sauerkraut. This process is the result of the combination of two types of fermentation by lactic acid bacteria (LAB) in a four steps process; pre-treatment, heterofermentation, homofermentation and maturation (Fleming et al., 1985; Roissart and Luquet, 1994).

Fermentation happens spontaneously by adding dry salt or brine to shredded cabbage under anaerobic conditions (Johanningsmeier et al., 2007). This fermentation grants a product that will vary depending of the indigenous microflora of the fields and the cultivar, thus procuring typical local flavors (Medina-Pradas et al., 2017). Spontaneous fermentation is simple and generally efficient but commercial sauerkraut requires pasteurisation or addition of chemical conservatives like sorbates and benzoates to stabilize the product (Fleming et al., 1985). This has led to the development of commercial starter cultures. The BLAC I starter culture used in this study has been developed 20 years ago to help standardizing the vegetable fermentations and avoid pasteurisation or chemical use to extend the shelf life of the products. The starter contains selected strains of *Lactobacillus plantarum*, *Leuconostoc mesenteroides* and *Pediococcus acidilactici* (Gardner et al., 2001). This starter has been succesfully used over the past two decades but has recently started to show an increased mortality over storage, this mortality could be related to the evolution of the indigenous microflora on cabbage that would be more competitive or by the presence of inhibitory components to the starter culture caused by the growing use of organic pesticides in the fields. The fermentation of organic cabbage with the BLAC I starter culture used to be more efficient but this may now be reversed.

The main problem of the fermentation industry is the spoilage caused by acid-tolerant yeasts able to use lactic and acetic acids under aerobic conditions and the residual sugars unused by the LAB without oxygen (Franco and Pérez-Díaz, 2012). This results in a secondary fermentation that may alter the stability, the organoleptic properties and the shelf life of the product by producing ethanol, carbon dioxide and reducing the acidity of the product (Moon, 1983; Thomas and Davenport, 1985). Previous works have identified two different yeast species (*Saccharomyces bayanus* and *Saccharomyces unisporus*) responsible of secondary fermentation in fermented carrots, cabbage and onions (Savard et al., 2002). The resistance of *S. unisporus* to acetic acid and lactic acid was so high that an addition of propionic acid was needed to inhibit its growth in an optimal media. On the other hand, *S. bayanus* had low resistance to acids but produced more gas (Savard et al., 2002).

The aim of this study was to follow the population of LAB and yeasts during the production of sauerkraut using cabbage produced under conventional or organic practices and to compare spontaneous fermentation to the use of a starter culture at both laboratory and industrial scale.

MATERIALS AND METHODS

Sauerkraut production

Sauerkraut fermentations have been carried out under three different variables: (1) conventional versus organic cabbage, (2) industrial (vats) versus laboratory (pails) scale, and (3) starter culture versus spontaneous fermentation. An industrial vat (600 kg) and three polypropylene pails (20 kg) were produced for each conditions tested from the same cabbage batch. Both type of cabbages of the cultivar *Brassica oleracea* variety *capitata f. alba* were bought in the same region and were conserved at 4 ^{o}C within use. Their outer leaves and cores

were removed. Then 320 kg of clean cabbage was grated and the following ingredients were added: water, sea salt (2% final concentration) and spices. Batches of 13 kg (repeated 24 times) were prepared, mixed and transferred into a stainless steel vat of 600 kg previously cleaned with hydrogen peroxide for 15 min and rinsed with tap water. When required, the rehydrated starter composed of *Lactobacillus plantarum*, *Leuconostoc mesenteroides* and *Pediococcus acidilactici* (BLAC I; Caldwell Bio Fermentation Canada Inc., Ste-Edwidge, QC, Canada) was inoculated at a final concentration of 6.5 log colony-forming units (CFU)/g (6 log CFU/g for each strain). The cabbage was then pressed to help breaking the vegetal cells and therefore extract sugars and remove a maximum of oxygen. At last, the vat was covered with two food grade plastic sheets with approximately 50 kg of chlorine water in between (Tolonen et al., 2002). Vats were incubated at 19 ^{o}C for 11 days before being transferred at 4 ^{o}C for a maturation phase (curring). This method is consistent with the optimum conditions for cabbage fermentation described by Pederson and Albury (1969). In parallel, food grade plastic pails of 20 kg were filled with 13 kg of cabbage, 4 kg of water and 340 g of salt and spices and carried out in triplicates to follow each batches at the laboratory scale (same conditions as the vats). They were closed using a plastic weight instead of the plastic sheets with static liquid weight to push the cabbage at the bottom, creating an air-liquid interface and at the top for the last day.

Sample collection and microbial counts

For every vat, three samples of 50 ml were periodically taken through a valve at the bottom of the vats and with a pipette at the surface by lifting lightly the plastic sheets covering vats to minimize the loss of the anaerobic environment created during the fermentation. In parallel, a sample of 50 ml was also periodically taken at the bottom of each pail with a syringe through a septum at four inches of the bottom.

Samples were then serially diluted into peptone water (1:10) and plated on plate count agar (PCA, Oxoid, Ottawa, ON, Canada) for aerobic and anaerobic total counts. For the microbial counts of LAB, deMan-Rogosa-Sharpe agar (MRS, Difco, Ottawa, ON, Canada) supplemented with 0.06% X-gal (BioShop Canada Inc., Burlington, ON, Canada) and 0.05% cycloheximide (Sigma, Saint-Louis, MO, USA) was used to distinguish the genus *Pediococcus* (white colonies), *Leuconoctoc* (blue colonies) and *Lactobacillus* (green colonies). Mayeux, Sandine and Elliker media (MSE, 10g tryptone, 5g yeast extract, 100g sucrose, 5g dextrose, 1g sodium citrate, 2.5g gelatin and 15g agar/L with 0.0075% sodium azide) was used as a validation of the *Leuconostoc* counts. Samples were also plated on *Petrifilm* (3M, London, ON, Canada) for coliforms and on Dichloran Rose Bengal Chloramphenicol (DRBC, VWR, Toronto, ON, Canada) for yeasts counts. Petri dishes were then incubated 48 to 72 h at 30 ^{o}C aerobically except for one series of the PCA that was incubated in anaerobic conditions. Cell counts were converted in log values and means were submitted to an ANOVA test. A difference between the treatments was determined at a significance level of 0.05. After the microbiological quantification, pH was measured for every sample, using a pH meter. Samples were then conserved at -20 ^{o}C for further quantification of sugars and organic acids by high performance liquid chromatography (HPLC).

Sugars and acids concentrations were determined using a HPLC system (Waters Unlimited inc., Toronto, ON, Canada) with an automatic injector 717+ at a flow of 0.4 ml/min. The system was coupled with a pre-column compatible with a column ION-300 (Concise Separations, San Jose, CA, USA) heated at 65 ^{o}C. The mobile phase used was 0.01 N H_2SO_4. The sugars (glucose, fructose and mannitol) and organic acids (lactic and acetic) as well as ethanol were detected respectively with a refractometer (2414-RI) at 40 ^{o}C and a UV detector (Water 2489_UV, Waters Unlimited Inc.) at 208nm. The software Empower 2 (Waters Unlimited Inc.) was used for integration.

Biomolecular analysis

Several colonies chosen from their morphology and color were purified by serial inoculation on selective medium with the goal of identifying predominant species during the fermentation. First, colonies of purified samples were transferred into 500 µl of dimethyl sulfoxide (DMSO, Sigma) in sterile microtubes. The tubes were incubated at 80 ^{o}C for 10 min then transferred in glacial ethanol at -80 ^{o}C for a rapid crystallisation of the cells. The thermal shocks were repeated three times before an incubation of 10 min at 65 ^{o}C. The tubes were centrifuged at 20,000 g for 5 min to remove the cell fragments. The supernatant was kept and 300 µl of isopropanol was added to precipitate the DNA overnight at -20 ^{o}C. DNA was then centrifuged at 20,000 g for 20 min (del Campo et al., 2010). The pellet was rehydrated in 20 µl of sterile water and stored at -20 ^{o}C until PCR and sequencing were performed using the Sanger method (Plateforme de séquençage et de génotypage; Université Laval, Québec, QC, Canada). For amplification of the 16S ribosomal RNA gene, primers used were 8f/fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r/rP1 (5'-GGTTACCTTGTTACGACTT-3') (Weisburg et al., 1991; Breidt et al., 2013a). For amplification of the internal transcribed spacer (ITS) region of the yeasts, primers used were ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') for an amplicon of 600 to 800 bp (White et al., 1990). Amplicon sequences were analysed using the BLAST program of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS

Comparison of sauerkraut fermentation with organic and conventional cabbage

As shown on figure 2.1, the fermentation of organic or conventional cabbage did not show any significant difference whether a starter culture was used or not regarding the main LAB counts. There were no significant variations ($P < 0.05$) regarding *Lactobacillus* and *Leuconostoc* species as they showed similar growth patterns both in conventional and organic cabbage. Moreover, the present study could not demonstrate other differences according to the pH or the microbial ecology. The only result showing variations between conventional and organic was about the coliform counts that disappeared faster in conventional than organic cabbage (1 and 5 days, respectively). In function of the absence of significant differences, results from conventional cabbage were not shown for the entire study and only the results from organic cabbage have been used to compare starter versus spontaneous and laboratory versus industrial scale.

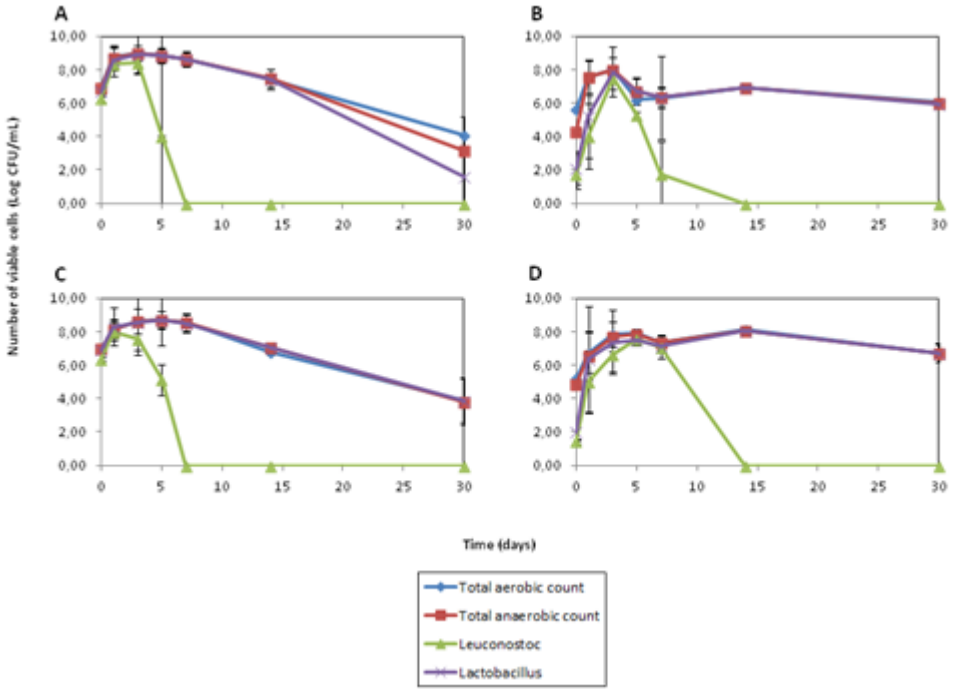

Figure 2.1 : Growth curves of the different microorganisms in the production of sauerkraut with organic cabbage and a starter culture (A), organic cabbage without starter (B), conventional cabbage with a starter culture (C) and conventional cabbage without starter (D). Viable counts are the mean of 3 samples at the bottom of the vats and 3 samples at the top. Error bars represent standard error on means.

Figure 2.2 presents the pH variation over 180 days as well as the delta pH (pH at t=0 – pH at t= 24 h) in the first 24 h of fermentation. In spontaneous fermentations, only a slight reduction of less than 0.5 units of pH is observed after the first day in the vats, while this variation is over 1.5 units when a starter is used. Moreover, the spontaneous fermentation vats needed up to 7 days to reach a pH value below 4.0, compared to less than 1 day (2 days in pails) with the starter culture. The final acidification was lower in spontaneous fermentations, as the pH stabilised around 3.71 to 3.87 and was more variable compared to a stable final pH lower than 3.50 with the use of a starter culture (Fig. 2.2). For pails, a little delay was observed in the acidification of the media in comparison with the vats. The initial acidification was slower in pails but did not influence the final acidification.

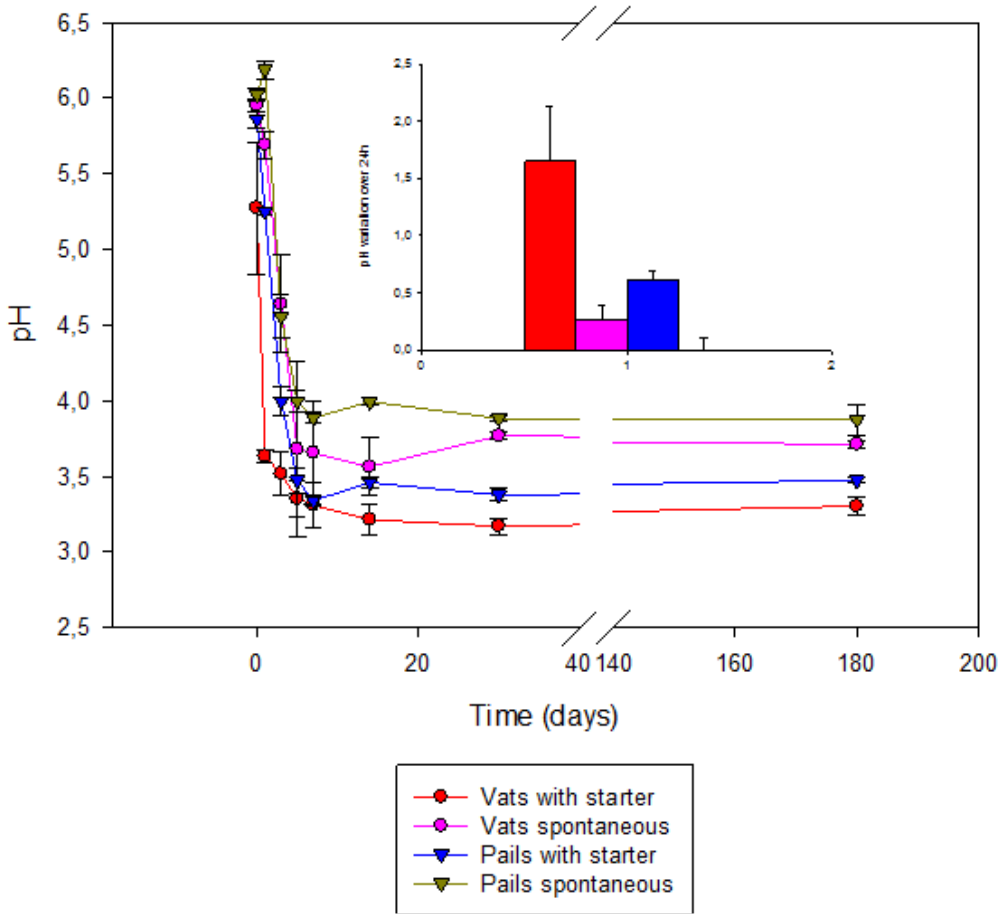

Figure 2.2 : Comparison of the evolution of the pH in spontaneous fermentation and with a starter culture at an industrial (vats) and laboratory (pails) scale. The pH values are the mean of 3 samples at the bottom and 3 samples at the top for the vats and 1 sample at the bottom of each pail. Error bars represent standard error on means.

Microbial ecology of the fermentations

In accordance with the delay in the pH reduction, the *Leuconostoc* population reached their maximal population slightly later in pails than in vats when a starter was used. The initiation of fermentation was always slower in spontaneous fermentation because of the lower initial LAB population (Figs. 2.2 and 2.3) as also described by Johanningsmeier et al. (2007).

Coliforms disappeared between 1 to 5 days and seemed not correlated with the use of a starter although less than 1 day was enough to replace most of the indigenous microflora with a starter while it took 3 days in spontaneous fermentations (Fig. 2.3). Aerobic and anaerobic total plate count (PCA) curves were very similar to the LAB counts on MRS, confirming the domination of those microorganisms (data not shown).

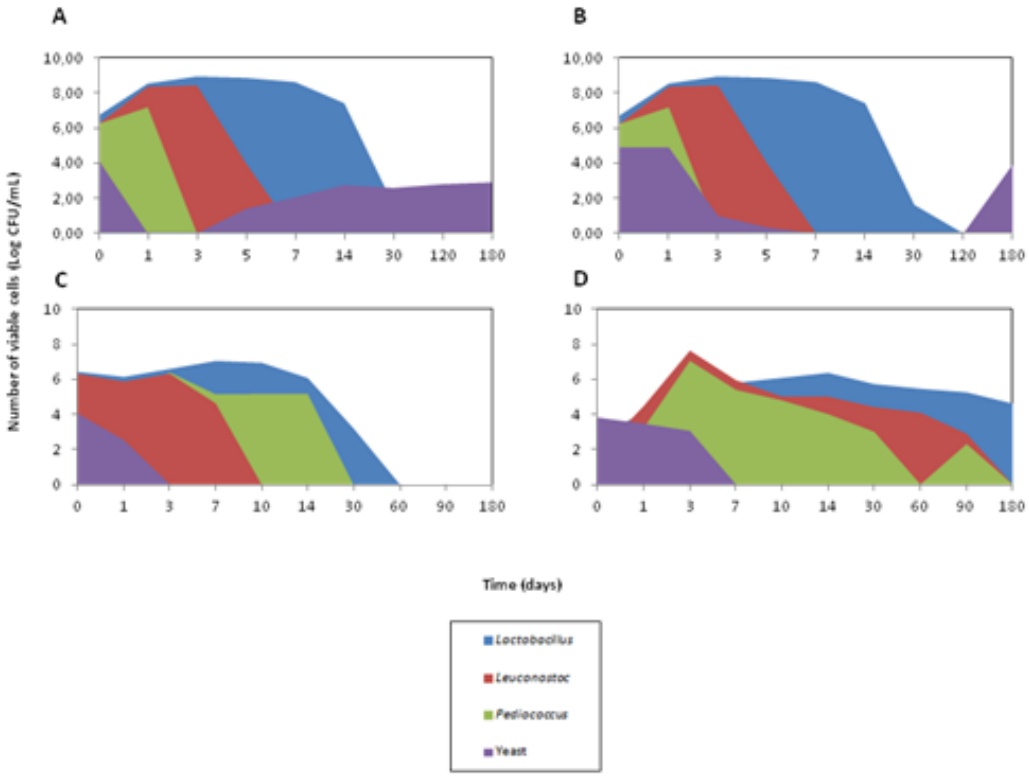

Figure 2.3 : Growth curves of the different microorganisms in the production of sauerkraut with organic cabbage in vats with a starter culture (A), in vats in spontaneous fermentation (B), in pails with a starter (C) and in pails in spontaneous fermentation. Viable counts are the mean of 3 samples at the bottom and 3 samples at the top for the vats and 1 sample at the bottom of each pail.

The *Leuconostoc* genus being responsible of the early stage of fermentation, or the heterofermentative phase, it grew rapidly in the first days (Fig. 2.3). Its viable counts reached up to 8 log CFU/ml within 24 hours or 6.5 log CFU/ml in pails when a starter culture was used (inoculum of 6 log CFU/ml), while it needed three days in a spontaneous fermentation to reach that level.

As shown on figure 2.4, *Leuconostoc* decreased progressively while *Lactobacillus* became the dominant genus reaching up to 7.0 to 8.5 log CFU/ml for the starter versus 5.5 to 7 log CFU/ml for spontaneous fermentation at day 7, and was always lower in pails. It caused the production of lactic acid (homofermentative phase) in high concentrations, which led to a higher acidification that inhibited the other LAB like *Ln. mesenteroides*. Mortality of the *Lactobacillus* occurred faster in the starter culture as the bacteria totally disappeared before 60 and 120 days in pails and vats, respectively (Fig. 2.4), leaving the yeasts as the only living microorganism. For spontaneous fermentations, a slow decrease was noted, but over 4 log CFU/ml of viable *Lactobacillus* remained after 180 days (Fig. 2.3). The high concentration of *Ln. mesenteroides* and *Lb. plantarum* made it harder to isolate *Pc. acidilactici* from the viable counts.

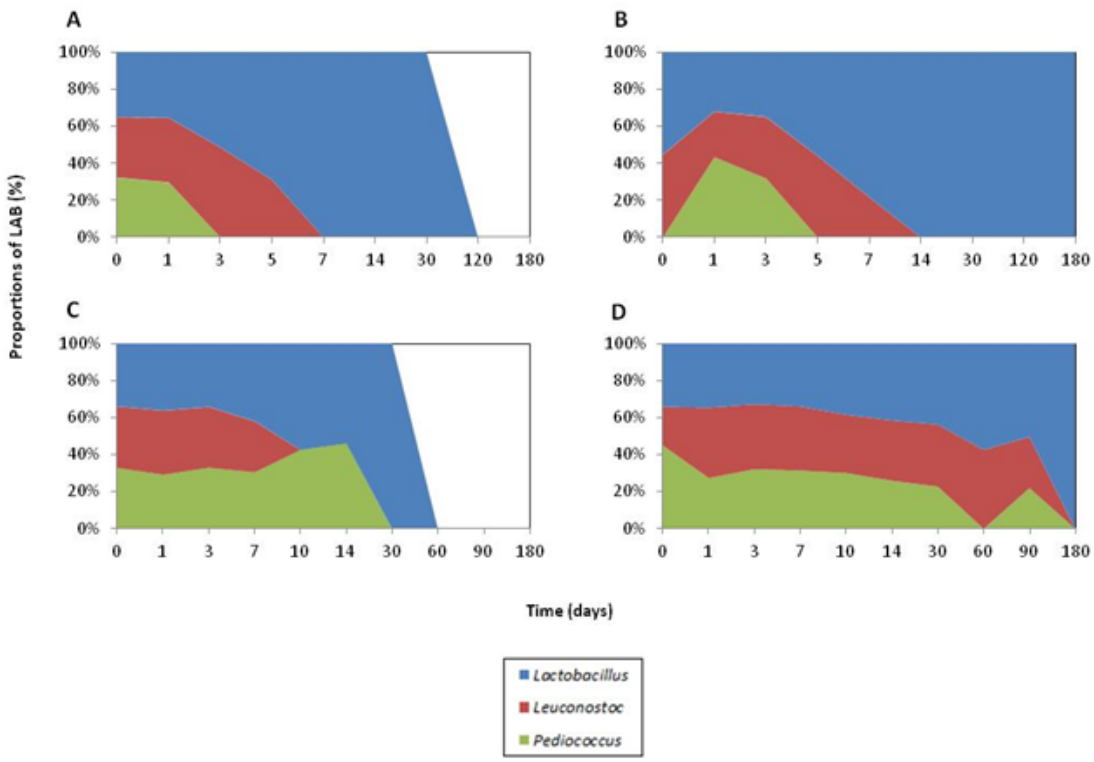

Figure 2.4 : Succession of the main LAB during the production of sauerkraut with organic cabbage in vats with a starter culture (A), in vats in spontaneous fermentation (B), in pails with a starter (C) and in pails in spontaneous fermentation. Viable counts are the mean of 3 samples at the bottom and 3 samples at the top for the vats and 1 sample at the bottom of each pail.

From fermentation in vats, a spatial separation has been observed early between the viable counts of LAB at the surface compared to the bottom. Higher viable counts of 1 log CFU/ml were noted at the surface in every condition (Fig. 2.5). This heterogeneity was observed faster when a starter culture was used in comparison to a spontaneous fermentation.

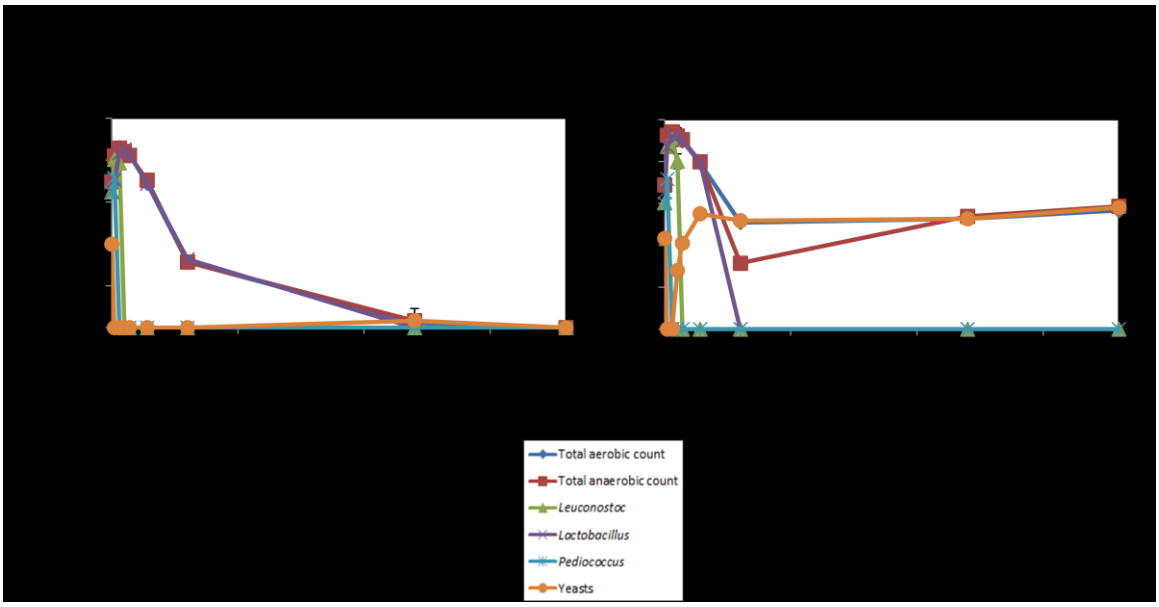

Figure 2.5 : Growth curves of the different microorganisms in the production of sauerkraut with organic cabbage and a starter culture. Viable counts are the mean of 3 samples at the bottom of the vats (A) or 3 samples at the surface (B). Error bars represent standard error on means.

Furthermore, at the beginning of the process, a high concentration of yeasts was observed (Fig. 2.6), reaching around 4 log CFU/ml in all conditions. However, the fermentation process caused a rapid decrease in the yeast populations, as most of the yeasts could not survive to the rapid acidification of the starter and even disappeared from the viable counts. However, some species reappeared (over the detection level) at the surface of the vats during the maturation phase.

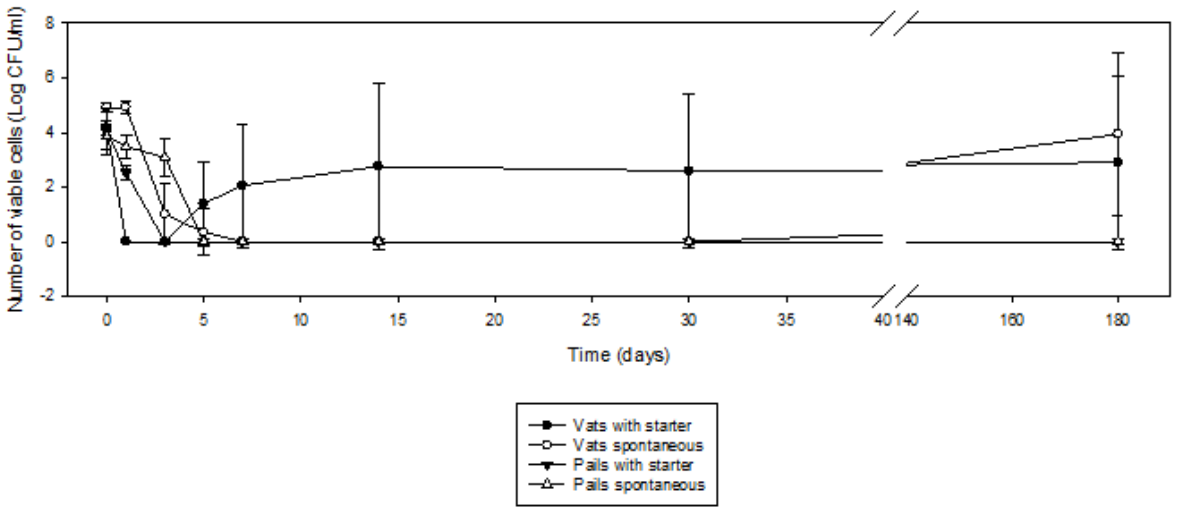

Figure 2.6 : Growth curves of the yeasts at the surface in the production of sauerkraut with organic cabbage under spontaneous fermentation and with a starter at an industrial (vats) and laboratory (pails) scale. Viable counts are the mean of 3 samples at the bottom and 3 samples at the top for the vats and 1 sample at the bottom of each pail. Error bars represent standard error on means.

Biomolecular identification

Since this study aimed to determine if the main LAB would be the same species in spontaneous fermentation and with the BLAC I, random colonies were chosen from the vats fermentations and identified with biomolecular analysis. Identification of species by sequencing of the isolated colonies from viable counts has allowed the identification of *Leuconostoc mesenteroides* as the only species of this genus in the vats containing a starter culture (Tab. 2.1). However, during the spontaneous fermentations, *Leuconostoc citreum* as well as *Lactococcus lactis* have been identified early in the process. The sequencing has also allowed the identification of *Lactobacillus brevis* in three of the vats and its colonies were visually identified on MRS plates for the last vat. This species was isolated until the end of the process even when a starter culture was used.

Table 2.1 : Identification of colonies randomly selected from the viable counts of each production at the industrial scale by sequencing of the 16S rRNA gene. The values correspond to the percentage of identification of each of the main species isolated and the total for all 4 vats.

Microorganisms	Organic with starter	Organic spontaneous	Conventional with starter	Conventional spontaneous
Leuconostoc mesenteroides	4	13	18	13
Lactobacillus plantarum	93	43	67	34
Pediococcus acidilactici	4	0	0	0
Lactobacillus brevis	0	17	3	31
Lactococcus lactis	0	4	0	6
Leuconostoc citreum	0	4	0	4
Other bacterial species	0	17	12	10
Kazakhstania servazzii	70	71	30	69
Pichia anomala	17	0	66	19
Other yeast species	13	29	5	13

Chemical analysis

Analysis of the samples by HPLC confirmed that the production of organic acids corresponded with the pH decrease and the growth of *Lb. plantarum* and *Ln. mesenteroides* as shown on figure 2.7. The same observation was noted with the production of ethanol and yeast growth (data not shown). Acetic acid was produced faster and in higher concentration early in the fermentation when the starter culture was used, therefore leading the process to a faster shift from the heterofermentation to the homofermentation phase.

Final concentration of acetic acid was reached after 3 days, as it took over 7 days in spontaneous fermentation (Fig. 2.7). For lactic acid, the production was also faster when the starter culture was used, but the production of this acid stopped around day 7 in every trial.

The final concentration of organic acids showed significant different final concentrations of acetic acid ($P < 0.05$) around 0.05% higher in spontaneous fermentations. On the other hand, lactic acid was generally present in higher concentrations with the use of a starter, reaching a higher concentration of 0.15 to 0.3% in comparison with spontaneous fermentations, thus explaining the pH differences (Fig. 2.7). These variations affected the lactic:acetic organic acid ratio in the final product. With a starter culture, it was between 5.5:1 and 6.5:1 as in spontaneous fermentation it showed slightly increased variations, from 2:1 to 3.5:1. A spatial heterogeneity was noted in the vats about the concentration of organic acids, following a similar pattern as the LAB. However, a homogeneization occurred over the maturation phase.

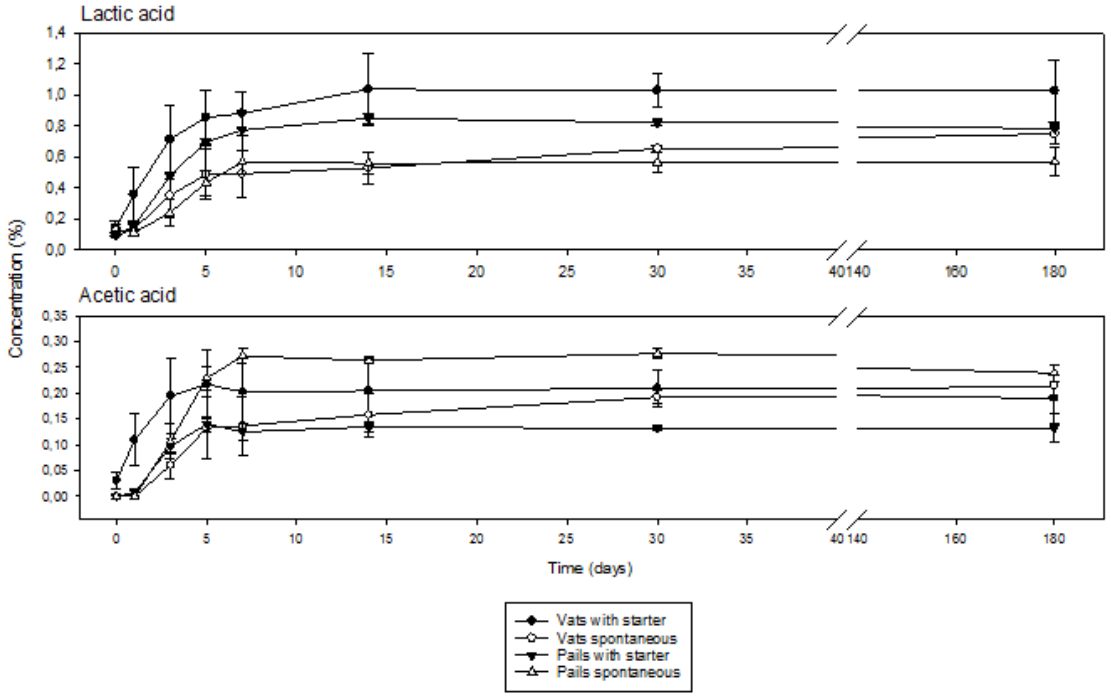

Figure 2.7 : Evolution of the organic acids concentration during the production of sauerkraut with organic cabbage under spontaneous fermentation and with a starter at an industrial (vats) and laboratory (pails) scale. The organic acid concentrations are the mean of 3 samples at the bottom and 3 samples at the top for the vats and 1 sample at the bottom of each pail. Error bars represent standard error on means.

For the main sugars, a gradual release of glucose and fructose from the vegetal cells was balancing the metabolism by LAB showing a very slow decrease over the process (Fig. 2.8). Glucose was metabolized to produce organic acids, as fructose was mainly transformed into mannitol during heterofermentation. No significant differences ($P > 0.05$) were observed between spontaneous and starter fermentations except that glucose was metabolized faster when the starter was used. At the end, the same amount of sugars was converted in acids in both type of fermentation. Also, nearly all the fructose was converted (0.2% remaining) to mannitol reaching a final concentration of 1%. Similar results were observed in pails, showing higher final concentrations of fructose (0.5%) and lower mannitol concentrations (0.5%) when a starter culture was used (Fig. 2.8). A concentration of less than 1% of residual glucose was observed at the bottom of the vats and in the pails, as this sugar was totally metabolized at the top of the vats probably resulting from the yeast growth.

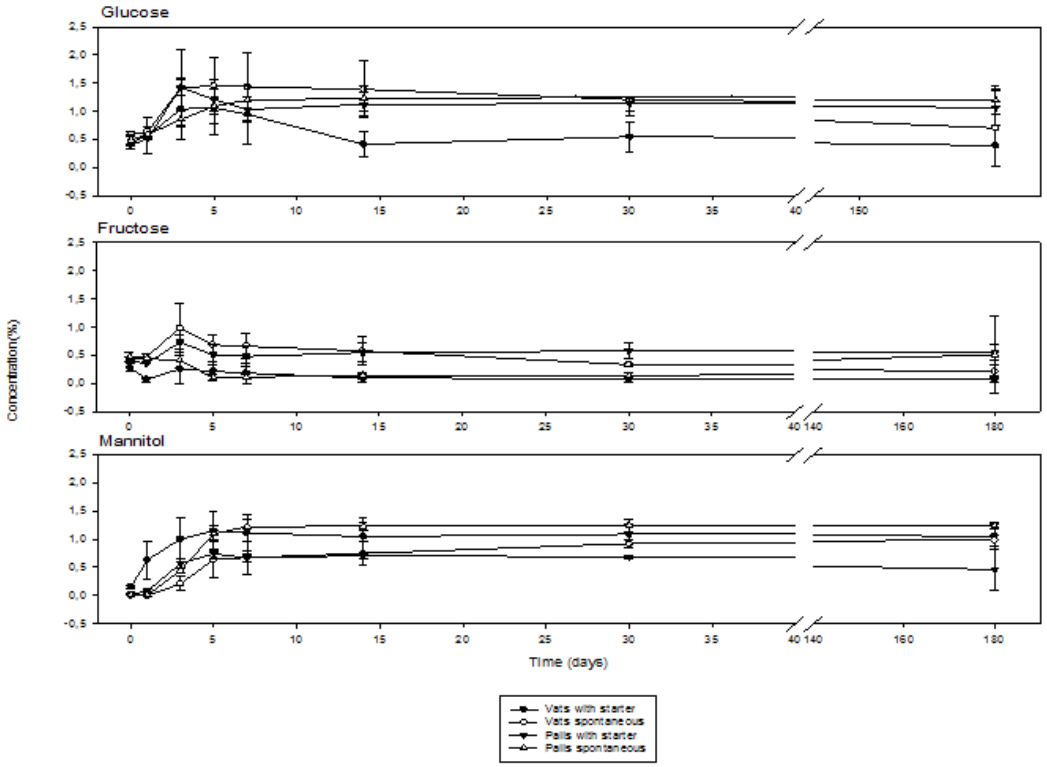

Figure 2.8 : Evolution of the glucose, fructose and mannitol concentrations during the production of sauerkraut with organic cabbage under spontaneous fermentation and with a starter at an industrial (vats) and laboratory (pails) scale. The sugars concentrations are the mean of 3 samples at the bottom and 3 samples at the top for the vats and 1 sample at the bottom of each pail. Error bars represent standard error on means.

DISCUSSION

Comparison of conventional and organic cabbage

The provenance of cabbage used for the different fermentation trials did not seem to influence the initial or the final acidification. This is consistent with the literature, since no major differences were noted by comparing organic to conventional cabbage. Bourn and Prescott (2002) reviewed most of the research and based on three main aspects (sensorial quality, nutritional value and safety), they could not define major impact about the mode of production. The only consistent and significant variation observed between conventional and organic cabbage would be the higher concentration of nitrites in conventional cabbage (Lieblein, 1993). Therefore, the main cause of variation would be due to the variation in the indigenous microflora that is influenced by the ground, climate and cultivar. This microflora is more important in spontaneous fermentations, as the starter culture could replace it rapidly as previously demonstrated by others (Wiander and Ryhänen, 2005; Beganović et al., 2014). In this study, no significant variations have been observed even in spontaneous fermentation. This could be explained by the use of the same cultivar for both conventional and organic cabbage. The only difference observed between the fermentation of organic and conventional cabbage was regarding the coliforms that survived longer in organic cabbage fermentation. This could simply be due to the presence of different coliforms in the fields where cabbage was grown, as no further identification of the strains was conducted in this study. However, Magkos et al. (2003) determined that there is no increased risk of pathogen contamination in organic vegetables. Therefore, we were unable to show any significant differences regarding the fermentation process.

Fermentation scale variations

The pails represented the fermentation occurring at the bottom of the vats as no samples have been taken at the surface of the pails except after 180 days of process. The main variations between laboratory and industrial scale were caused by the height of the vats and the pressing setup (static block versus liquid weight). Since the vats were sampled at the top and the bottom, it allowed the observation of a spatial separation early in the process (from day 1 to the maturation phase). Effectively, during the heterofermentative phase, the production of carbon dioxide could have created a flow towards the top of the vats and pails, carrying the microorganisms and sugars. Therefore, samples from bottom and surface of the vats were analysed separately and significant higher viable counts of approximately 1 log CFU/mL were observed at the surface during the heterofermentation phase. This is not linked with the growth rate as curves were very similar for both sampling spots in vats. Although a difference in the LAB concentration was rapidly created between the top and the bottom of the vats, a slow homogenisation was observed in spontaneous fermentations during the maturation at 4 ^{o}C. The literature offers very little information about such spatial variations in vegetable fermentations. However, Kraut-Cohen et al. (2016) observed different zones of varying microbial ecology in commercial silages from bunker silos which is in agreement with our results.

The main difference between fermentation in vats and pails was about the yeasts growth. These larger microorganisms (10-100 times larger than bacteria) can cause major problems in food fermentation and particularly in vegetables. Yeasts can survive under acidic conditions and then generate a secondary fermentation or spoilage fermentation as described by Breidt et al. (2013b). In the industrial vats, it was noticed that the yeasts disappeared from the viable counts between day 3 and 5 at the bottom, but persisted at the surface or reappeared over the detection level during the maturation phase. The main hypothesis to explain this observation is linked, once again, to carbon dioxide produced by heterofermentative bacteria leading the yeasts to the surface of the vats. Following this movement, there would be formation of air

pockets under the plastic sheet covering the vats. That could lead to the apparition of less acidic microenvironments. Those microenvironments would be favourable to the formation of biofilm at the air-liquid interface, thus reducing the efficacy of organic acids, as biofilm cells are significantly more resistant to planktonic cells (Bridier et al., 2011; Brugnoni et al., 2012). Biofilms causes major problems in many industries of food processing and are very hard to clean (Shi and Zhu, 2009; Srey et al., 2013). The acid-tolerant yeasts could then survive, adapt, grow and contaminate the whole area, being redistributed during the packaging. Many studies have demonstrated the existence of persistent cells which are more resistant to the environment conditions than regular cells and who will be contributing to the viability of the strains in the biofilm (Olszewska, 2013). It was also demonstrated by Didienne et al. (2012) in salers cheese that viable cells in biofilms attached to the vats sidewalls can act as a starter culture in the following fermentations. Those persisting microorganisms are a cause of post-processing contamination (Oliveira et al., 2010; Tomičić and Raspor, 2017). Further studies will be needed to confirm this theory by the detection and characterization of potential biofilms but preliminary results tend to confirm this hypothesis.

On the other hand, none of the 12 pails of 20 kg has shown fungal contamination after 180 days of process as for the samples taken at the bottom of the vats. Remember that in these pails, the cabbage was pressed with a plastic weight and not covered by a plastic sheet. This may have contributed to avoid the possible formation of microenvironments favourable for the survival and the development of yeasts and biofilms at the surface. These observations support the hypothesis that biofilm would have been developed over the years at the top of the stainless steel vats, helping the yeasts to survive to the early acidification and allowing them to slowly develop a higher resistance to organic acids and low pH conditions. Since the same species were identified in all vats, the yeast persistence could be directly due to the presence of fungal biofilm contaminating the subsequent sauerkraut productions as observed by Didienne et al. (2012) in cheese production.

Only two species have been isolated late in the fermentations, those two yeasts have been identified as *Kazachstania servazzii* and *Pichia anomala*. The first one is a close species to the yeasts found by Savard et al. (2002). As *Pichia anomala* is known for its killer activity, it has a potential to act as a biocontrol agent for other yeasts and moulds such as *Saccharomyces cerevisiae*, *Aspergillus candidus* and many others (Petersson et Schnürer, 1995; Fredlund et al., 2004; İzgü et al., 2006). Therefore, it is possible that *P. anomala*'s selection over the other yeasts species was directly favored by its toxin production (Champagne et al., 2017). Further studies of these yeasts and presence of biofilm will be needed to confirm these hypotheses. Some of the main spoilage yeasts in fermented vegetables are already known as *Debaryomyces hansenii*, *Saccharomyces cerevisiae*, *Kazakhstania servazzii*, *Zygosaccharomyces rouxii* and *Pichia anomala* (Deak, 2007). Overall, at the exception of those observations, a normal fermentation process occured, as *Leuconostoc* and *Lactobacillus* genus dominated the heterofermentative and homofermentative phases respectively.

At the end of the maturation phase, residual glucose was found at the bottom of the vats and in the pails, but not at the top of the vats. This can be explained by the high growth of yeasts at the surface of all the vats during the maturation phase. There was no such variation in the concentration of mannitol since yeasts could not metabolize it. Effectively, yeasts can use glucose but not mannitol at acidic pH (Barnett, 1976). The production of mannitol during the heterofermentative phase is important to reduce to a minimum the residual sugars that can be used by yeasts to cause spoilage (Rodríguez et al., 2012). A fairly sufficient sugar metabolism of the starter culture removes the need for pasteurization of the sauerkraut (Gardner et al., 2001).

Comparison of spontaneous fermentation to a starter culture

In fermentation with a starter culture, the results have shown that one day was enough to replace the indigenous microflora while it took three days in spontaneous fermentations. This confirms that the use of a starter allows a better control of the fermentation process. It has been noted from past experimentations that a fast growth of LAB favors a faster decrease in pH and therefore inhibits both the pathogens and the spoilage microorganisms responsible for production of toxins and degradation of the cabbage, respectively (Gardner et al., 2001; Viander et al., 2003). The rapid initiation of the fermentation caused by the starter reduces the time gap for undesirable microorganisms to have negative effects during the early stage of fermentation, thus improving the organoleptic qualities and appearance of the final product (Viander et al., 2003; Johanningsmeier et al., 2007).

The starter culture was very efficient during the fermentation phases of the process. However, it demonstrated a weakness over the maturation phase, which is illustrated by the accelerated mortality of the LAB. Two main hypotheses can explain this situation. The first one can be related to the high efficacy of the starter culture itself. Since it leads to a high and very fast acidification, producing lactic acid faster than spontaneous fermentation, *Lactobacillus* species would be unable to adapt to the conditions of the media because of the organic acid ratio produced. Effectively, if the pH is lower, more acetic acid will not be dissociated and therefore act as an antimicrobial compound. Combined with the fact that more acetic acid is produced early in the fermentation with a starter, the *Lactobacillus'* viability could be affected (Wiander and Ryhänen, 2005). Secondly, it has also been reported by Lu et al. (2003) that bacteriophages may have a role in the succession of the species from the heterofermentation to the homofermentation phase of the process and that there is a complex microbial interaction between the LAB and the bacteriophages in sauerkraut production (Lu et al. 2003). Therefore, there could be a specific evolution of bacteriophages targeting the strains of the starter culture while indigenous strains would be less or non-affected in spontaneous fermentations.

However, these two hypotheses were not validated during this study. For spontaneous fermentation, a low decrease in the LAB population was observed, in accordance with the results of Viander et al. (2003).

As *Pc. acidilactici*'s growth was very low and its viable counts decreased rapidly in the fermentation, the utility of this species in the starter culture was not demonstrated for this range of temperature (19 ^{o}C). This information suggests that this strain could be replaced by a new species with faster growth and better resistance to organic acids. However, it is believed that for fermentations without temperature control, *P. acidilactici* may play a role in the process (Medina-Pradas et al., 2017).

Biomolecular identification of the isolated strains allowed the observation of a greater diversity of LAB in spontaneous fermentations which demonstrates the higher control and stability of the process with the use of a starter culture. It was not surprising to find a great variety of species since such molecular ecology diversity was already reported by Breidt (2004). *Leuconostoc citreum* has been described as genetically very similar to *Ln. mesenteroides* (Farrow et al., 1989) and has been already isolated in sauerkraut and kimchi (Jung et al., 2011). *Lactococcus lactis* is a heterofermentative bacteria that has already been isolated from Chinese sauerkraut on another study (Xiong et al., 2014). The most interesting finding was *Lactobacillus brevis* that could grow enough to be detected on the viable plate counts even when a starter culture was initially added at a rate of 6.5 log CFU/ml. This means that the starter was not able to fully control the fermentation process or, at least, this species. Also, *Lb. brevis* is known to have great probiotic properties and could be interesting to insert in a new mixed microbial starter culture (Beganović et al., 2014). Further studies about its heterofermentative fermentation pattern and its capacity to survive during the maturation of sauerkraut should be evaluated (Roissart and Luquet, 1994). Although it is known that *Lb. brevis* can terminate the fermentation of sauerkraut, reaching a lower final pH in the media

(Medina-Pradas et al., 2017). However, incorporating it in a starter culture could jeopardize the role of the other species.

The use of a starter culture standardized the microbial growth which directly leads to a better control of the organic acids production (Beganović et al., 2014). The main differences between spontaneous fermentation and the use of a starter are the rapid acidification and the stability of the organic acid ratio in the final product. When a starter culture was used, low variations were observed in comparison with the spontaneous fermentation since this process mainly depends on the unstable indigenous microflora of raw cabbage. Such variation is not good for the industry that expects standardized final products. It was already demonstrated that using a starter culture helps standardizing the process (Font de Valdez et al., 1990). It has also been reported that a lactic:acetic acid ratio between 3.5 and 5.0 is needed to obtain good sauerkraut quality (Pederson and Albury, 1969). This ratio's importance has been confirmed for the organoleptic qualities of the sauerkraut since sensory analysis demonstrated that the starter culture was preferred to spontaneous fermentation by an evaluation panel (Martinez-Villaluenga et al., 2012). Therefore it also confirms the importance of controlling the different fermentation phases with a mixed starter culture to obtain a final product with the right concentration of each organic acid as it has been described by Gardner et al. (2001). The results obtained in this section clearly demonstrate the efficiency of starter cultures as reported by others (Font de Valdez et al., 1990; Johanningsmeier et al., 2007).

CONCLUSION

Mainly, the results of this study have demonstrated the absence of significant differences between the microbial ecology of the fermentation of organic and conventional cabbage, the differences being only related to the provenance of the cabbage and its indigenous microflora and therefore only influenced the beginning of spontaneous fermentations. This study also

demonstrated the importance of the use of a starter culture to obtain a standardized high quality end product with a good ratio of organic acids. The spontaneous fermentations showed greater variations since the fermentation phases were not controlled. The main difference observed between laboratory and industrial scale was the growth of yeasts in the vats at the surface.

This study showed the problem of spoilage yeast contamination in the actual process used by the industry. The starter culture and spontaneous microflora seems unable to completely inhibit the growth of acid-tolerant yeasts. This could be due to the presence of biofilms at the air-liquid interface although data obtained with pails are suggesting that the sealing of vats with plastic sheets may be promoting the problem. The starter culture BLAC I demonstrated an early mortality of most of the LAB during the maturation phase which would be due to its capacity to produce very high concentrations of organic acids over a short period of time, fragilizing the bacteria themselves. An update of the starter culture could help to extend the viability of LAB during the maturation. *Lactobacillus brevis* seems a promising addition to a starter culture as it demonstrated a high growth speed and viability and is known to be more resistant to organic acids than *Lactobacillus plantarum* (Medina-Pradas et al., 2017). The importance of the maturation phase was also demonstrated during the process, since an important separation between the top and the bottom is rapidly created during the fermentation in industrials vats.

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CHAPITRE 3

FORMATION DE BIOFILMS PAR LES LEVURES ACIDO-RÉSISTANTES CONTAMINANT LES PRODUCTIONS INDUSTRIELLES DE CHOUCROUTE EN CUVES D'ACIER INOXYDABLE

Lors de cette étude, la croissance de biofilms fongiques sur une paroi en acier inoxydable a été investiguée sous différentes conditions de fermentation de choucroute. La capacité des levures à produire un biofilm durant la fermentation lactique a été évaluée sur des coupons en acier inoxydable à l'aide de réacteurs CDC. Les principales levures contaminant les productions de choucroutes industrielles on été isolées à partir des parois de cuves en acier inoxydable et ont été identifiées comme étant *Kazakhstania servazzii* et *Pichia anomala*. Ces deux espèces ont été inoculées en cultures pures et en co-culture dans des matrices de jus de choux en fermentation spontanée et avec un ferment. La microscopie électronique et les dénombrements de cellules viables ont été utilisés pour suivre le développement du biofilm fongique sous l'influence de la fermentation des bactéries lactiques sur le développement. Cette étude a permis de confirmer la capacité des levures isolées en fermentation industrielle à former un nouveau biofilm à l'échelle laboratoire. Les résultats semblent aussi démontrer que les levures ont une préférence spatiale pour la production de biofilm à l'interface air-liquide, puisque celles-ci ne sont pas complètement contrôlées par la fermentation même lorsqu'un ferment est utilisé.

La contribution des auteurs a été la suivante : j'ai écrit les protocoles, puis effectué la totalité des manipulations et analysé les résultats avec le support de Caroline Lapointe. Les protocoles et manipulations en microscopie électronique ont été faits par Denise Chabot. Pour la rédaction, j'ai écrit les premières versions de l'article avec les révisions des Dr Tony Savard et Dre Carole Beaulieu. Les objectifs et le projet ont été rédigés par le Dr Tony Savard.

ACID-TOLERANT YEASTS CAN FORM BIOFILMS ON STAINLESS STEEL INDUSTRIAL VATS IN SAUERKRAUT FERMENTATION

Yanick Bisaillon [a, b], Caroline Lapointe [a], Denise Chabot [a], Carole Beaulieu [b], Tony Savard [a]

[a] *Agriculture and Agri-Food Canada*

[b] *Université de Sherbrooke*

ABSTRACT

In this study, the growth of fungal biofilm on stainless steel in different sauerkraut fermentation conditions was investigated. Yeasts capacity to produce biofilm during lactic acid fermentation was evaluated on stainless steel coupons with the use of CDC Biofilm Reactors. The main contaminating yeasts were isolated on the sidewalls of stainless steel vats during and after industrial-scale fermentations and were identified as *Kazachstania servazzii* and *Pichia anomala*. Both species were grown separately and in co-culture under spontaneous fermentation and with a starter culture. Scanning electron microscopy and viable counts were used to determine the influence of lactic acid bacteria fermentation on fungal biofilm development. This study confirmed that yeasts isolated from the surface of industrial-scale vats are able to create biofilm on stainless steel at laboratory scale. The results also suggest that yeasts have a spatial preference for the production of biofilm at the air-liquid interface and are not completely controlled by the fermentation of lactic acid bacteria, whether a starter culture is used or not.

INTRODUCTION

Biofilms are an assemblage of extracellular polymeric substances produced by microorganisms attached to a surface (Sauer et al., 2007). The matrix protects the microorganisms from the environment but allows the diffusion of nutrients for the microbial growth. Therefore, biofilms are problematic for food processors because their structure increases the resistance of microorganisms to cleaning and disinfection (Shi and Zhu, 2009). Moreover, subsequent contamination may be more likely to occur if the biofilm structure is not removed during sterilization processes. Failure to remove the structure could allow a faster adhesion of future microorganisms into the existing biofilm (Timke et al., 2008; Shi and Zhu, 2009).

Even though the first report on biofilms was published nearly 75 years ago, these aggregates remain one of the main causes of contamination problems encountered in the food processing industry (Zobell, 1943). Several strategies have been deployed to control biofilm development, from simple cleaning and disinfection to the use of enzymes, phages and metabolites (Simões et al., 2010). Despite all those strategies and the knowledge that has been acquired, biofilms still cause contamination by foodborne pathogens in the poultry, dairy and meat industries in particular, thus threatening the health of consumers (Lindsay et al., 2006). Persisting microorganisms also promotes the risk of post-processing contamination by spoilage microorganisms, which leads to considerable industrial losses (Shi and Zhu, 2009; Oliveira et al., 2010; Tomičić and Raspor, 2017).

The capacity of microorganisms to develop biofilm and adhere to the surface is highly dependent of the type of surface, whether they are hydrophilic or hydrophobic, the texture of the surface, the temperature, the nutrient content of the medium, and the microbial species involved (McCourtie and Douglas, 1981; Stepanović et al., 2004; Brugnoni et al., 2007;

Bürgers et al., 2010; Nguyen et al., 2011). The most commonly used surface in the food industry is stainless steel because its hydrophilic nature makes it one of the most efficient surfaces to resist biofilm formation (Midelet and Carpentier, 2002; Myszka and Czaczyk, 2011; Moreira et al., 2015). However microorganisms are still able to adhere to this material, depending on the finish of the surface (Arnold and Bailey, 2000).

Among microorgansims that adhere to stainless steel, many studies have shown the yeast capacity to produce biofilm (Tomičić and Raspor, 2017). In the food industry, *P. anomala* has been considered as a spoilage microorganism being capable to produce biofilm on stainless steel in the beverage industry (Tristezza et al., 2010; Brugnoni et al., 2012). Furthermore, Moen et al. (2015) demonstrated that, for studies of the development of biofilms on stainless steel, the use of stainless steel coupons are a good model to represent the food industry.

In this study, the development of fungal biofilm caused by spoilage yeasts was investigated during the production of fermented vegetables, specifically sauerkraut. In a previous study, the microbial ecology of sauerkraut production was investigated at an industrial scale. ITS region sequencing of isolated yeasts allowed the identification of *Pichia anomala* and *Kazachstania servazzii* as the most abundant species. Those yeasts managed to survive through the whole fermentation process and over the maturation phase. The presence of fungal biofilm in sauerkraut production has been, to our knowledge, poorly documented. However, the presence of yeasts during the fermentation process suggests that yeast biofilm could be produced at the air-liquid interface. It was proposed that gas production during heterofermentation can create a positive flow from the bottom to the surface, leading to a higher yeast concentration in that region. The yeasts would then be trapped in existing biofilms or in the folds of the plastic sheet used to cover the vats, creating less acidic microenvironments. The yeasts would add to existing biofilms or produce new biofilm at the air–liquid interface and survive until the end of the fermentation process before growing and contaminating the surface during the maturation phase (Bisaillon et al., in preparation).

The spoilage of fermented foods by yeasts can occur in two different patterns. First, during their growth, yeasts can produce carbon dioxide and ethanol that will have an impact on the organoleptic qualities of the final product (Moon, 1983; Thomas and Davenport, 1985). Secondly, yeasts can use acetate and lactate produced by lactic bacteria as a carbon source in the presence of oxygen leading to an increase in the pH of the medium, thus reducing the hurdles to control the growth of pathogens and other spoilage microorganisms, particularly in the case of a post-processing contamination (Franco and Pérez-Díaz, 2012).

The two main contaminating yeasts identified have already been studied for their role in food processing. *K. servazzii*, that was predominant in sauerkraut production, has also been identified as a spoilage microorganism in kimchi, another fermented vegetable product, causing swelling problems (Moon et al., 2014; Spanoghe et al., 2017). The other predominant yeast species that was associated with sauerkraut production, *P. anomala,* does not appear to be a threat to food quality. This yeast has been also isolated from various habitats including food fermentation and plays a role in flavour enhancement of fermented product (Walker, 2011). *P. anomala* is however mainly interesting for its killer activity. Several studies reported its capacity to act as a biocontrol agent against the growth of other yeasts and moulds such as *Saccharomyces cerevisiae* and *Aspergillus candidus* (Petersson and Schnürer, 1995; Fredlund et al., 2004; İzgü et al., 2006; Tao et al., 2011). Moreover, Savard et al. (2005) have incorporated *P. anomala* in a starter culture to inhibit two *Saccharomyces* species contaminating fermented vegetables.

The aim of this study was to confirm the presence of biofilm in industrial stainless steel fermentation vats used for sauerkraut production. Furthermore, it was aimed to determine the impact of lactic acid bacteria (LAB) fermentation on fungal biofilm in cabbage juice in spontaneous conditions and with the use of a starter culture. The capacity of yeasts isolated at the industrial scale to produce new biofilms at laboratory scale was also investigated.

MATERIAL AND METHODS

Biofilm detection

In a previous study, the microbial ecology of sauerkraut production was studied in four industrial stainless steel vats. In the present study, the surface of those stainless steel vats was sampled for residual viable microorganisms in biofilm. The samples were taken using sterile scrubbing pads and razor blades as soon as the vats were emptied. The vats were sampled at the top (air-liquid interface) and at the middle of their height. The tools were then incubated into yeast and mould (YM) broth (Difco, Montréal, Québec, Canada) and deMan, Rogosa and Sharpe (MRS) broth (Difco) (Grounta et al., 2015). Initial viable counts were determined after the tools had been vigorously shaked into the media by hand or with a vortex mixer for at least 10 s. The broths were then incubated up to 10 d at 19 ^{o}C and microorganisms were isolated on MRS agar, YM agar and plate count agar (PCA, Difco). Colonies were selected, isolated and purified for identification by sequencing the 16S rDNA gene for bacteria or internal transcribed spacer (ITS) region for yeasts. For the identification, DNA extraction and amplification were achieved similarly as described by del Campo et al. (2010), using ITS1 and ITS4 primers (White et al., 1990). Sequencing was performed using the Sanger method (Plateforme de Séquençage et de Génotypage; Université Laval, Québec, QC, Canada). Sequencing data were analysed using the BLAST tool of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the species.

Strain selection for biofilm growth essays

Of the 97 isolated and identified yeast strains, 49 were detected during a previous study about the microbial ecology of sauerkraut production and are described in Table 2.1

(Bisaillon et al., in preparation). Another 48 strains were isolated during this study and are described in Table 2.1. A total of 29 yeast strains were chosen for further analysis of their spoilage and biofilm capacities. The strain selection process made sure that at least one of each identified species was selected and that the highest diversity in the origin of the strains with regard to the vat, method and time of isolation was maintained.

All the selected strains were tested for gas production in cabbage juice and sauerkraut juice with 2% NaCl at 4 and 27 ^{o}C to represent conservation and optimal temperatures, respectively. Cabbage juice was produced using a Santos Juice Extractor 28 (Santos, Lyon, France) and conserved at -20 ^{o}C whitin use. Then, for the evaluation of gas production, 10 mL of cabbage juice or sauerkraut juice was placed in tubes containing an inverted Durham tube sterilized 3 min at 110 ^{o}C. Cultures of each strain were grown in YM broth 48 h at 27 ^{o}C. Media was inoculated in triplicate at a rate of 1% with a fresh culture standardized at 8 log colony-forming units (CFU)/ml and were incubated until the Durham tubes were full of gas or for up to 45 d. Gas production was evaluated every 24 h over the first 7 d and then every 48 h.

The strains were also tested for their capacity to produce biofilm in cabbage juice. Pure cultures incubated for 48 h at 27 ^{o}C were centrifuged for 20 min at 4400 g and suspended into 0.85% saline twice. Then 10 ml of cabbage juice was inoculated at 1% with the washed culture, and 2 ml was transferred in a 12-well microplate, where sterile glass slides were inserted (half of each slide was covered by the medium). The plate was then closed and incubated at 19 ^{o}C for 72 h. After the incubation, the slides were rinsed three times in 0.85% saline before being transferred into a 50-mL sterile tube containing 10 mL of 0.1% (w/v) Bacto peptone (BD, Mississauga, ON, Canada) and 0.1% (v/v) Tween 80 (Sigma, Saint-Louis, MO, USA). In order to remove biofilms, tubes were alternately vortexed for 30s (three times) and sonicated 4 min (two times) (Lapointe et al., submitted). Lastly, biofilm and planktonic cell counts were obtained by serially diluting the samples in 0.1% peptone water (1:10) and inoculating YM agar using the spread plate method. The experiment was carried out in

triplicate for each strain, and the viable counts from biofilms were used to determine the best biofilm-producing strains.

Biofilm production in CDC Biofilm Reactors

From the best biofilm producers, selected yeasts were furthermore characterized for their biofilm production in four different conditions: (a) an inoculum of 4 log CFU/ml of yeast with a commercial starter culture for the fermentation of vegetable (BLAC I starter culture; Caldwell Bio Fermentation Canada Inc., Ste-Edwidge, QC, Canada) inoculated at 6.5 log CFU/ml in sterile cabbage juice (3 min at 110 ^{o}C); (b) an inoculum of 4 log CFU/ml of yeast in spontaneous conditions (without starter culture); (c) an inoculum of 2 log CFU/ml of yeast with the starter culture BLAC I inoculated at 6.5 log CFU/ml in sterile cabbage juice; and (d) an inoculum of 4 log CFU/ml of yeast in sterile cabbage juice without starter. In all conditions, the cabbage juice was diluted 3:4 in deionized water to reduce the inhibitory effect of potential bioactive compounds. Four CDC Biofilm Reactors (BioSurface Technologies Corp., Bozeman, MT, USA) containing eight rods each were used in parallel, so that trials of the three selected yeast strains in pure cultures and a mixed culture of both yeast species were conducted at the same time. CDC Biofilm Reactors were used accordingly to the ASTM E2562 - 07 book of standards (ASTM, 2007).

First, the CDC Biofilm Reactors were prepared and sterilized for 30 min at 121 ^{o}C as described by ASTM (2007), and then 350 mL of cabbage juice was added to reach the level of half of the top stainless steel coupons, since each rod held three coupons. The reactors were then inoculated at 1% with the standardized yeasts and bacterial cultures depending on the conditions tested and were incubated at 19 ^{o}C for 10 d without agitation.

Samples were taken 2 h after inoculation and on days 1, 4, 7 and 10. Every time, a rod was removed and replaced by a sterile one, and 5 mL of the medium was collected to determine planktonic cell counts. Viable counts for LAB were obtained from the spread plate method on MRS agar supplemented with 0.006% X-gal (BioShop Canada Inc., Burlington, ON, Canada) and 0.05% cycloheximide (Sigma). Then, YM agar supplemented with 0.02% chloramphenicol (Sigma) and 0.03% tetracycline (Sigma) was used to inhibit bacterial growth in order to obtain specific yeast counts. The coupons were removed from the rods and treated in accordance with the same method as the glass slides to remove the potential biofilm. All the conditions were carried out in triplicate. Cell counts were converted in log values, and means of three trials were submitted to an ANOVA test. A difference between the treatments was determined at a significance level of 0.05.

Scanning electron microscopy

At the end of the trials with the mixed yeast culture, a rod was kept for scanning electron microscopy (SEM) and treated following an adaptation of the protocol of Oliveira et al. (2010). The stainless steel coupons were rinsed in sterile 0.85% saline and washed three times in 0.1 M cacodylate buffer (CB), pH 7.2, for 15 min. Biofilms were then fixed using the CB supplemented with 2.5% glutaraldehyde and 0.15% ruthenium red for 2 h and then rinsed with CB once and then water twice for 15 min each. A second fixation was performed with 1% osmium tetraoxyde for 1 h then rinsed three times with water. The coupons were then serially dehydrated with 30%, 50%, 70%, 80%, 90%, 95%, and 100% ethanol and kept in a solution of 100% ethanol so that they could be sent for structure analysis. The coupons were then rinsed with 100% ethanol, dried with critical point dryer (Biodynamics Research Corp., Rockville, MD, USA) and covered with 7 nm of gold before observation with a scanning electron microscope (Quanta 600; FEI Company TM, Brno, Czech Republic).

RESULTS AND DISCUSSION

Biofilm detection and species proportions

After fermentation, yeasts and LAB were isolated by scrubbing the inner walls of the vats at the air-liquid interface and at the middle of their height. *K. servazzi* was identified more often in the sauerkraut juice during fermentation (71% of the isolates) (Table 3.1), while *P. anomala* was more dominant in the biofilms (50% of the isolates) (Table 3.1). The presence of microorganisms on the vats sidewalls demonstrates that there is an attachment to the surface that is strong enough to withstand fermentation and industrial cleaning. Similar techniques were also used in other studies to demonstrate the presence of biofilm (Timke et al., 2008; Grounta et al., 2015).

Table 3.1 : Number of isolates of the different yeasts in the sauerkraut process during the fermentation and maturation and after fermentation on the vats sidewalls.

Species identified	Number of isolates during fermentation	Number of isolates from the vats sidewalls	Total
Kazachstania servazzii	35	15	50
Pichia anomala	12	24	36
Meyerozyma guilliermondii	1	3	4
Galactomyces geotrichum	1	0	1
Galactomyces candidum	0	1	1
Pichia fermentans	0	1	1
Trichosporon spp.	0	4	4

Strain selection

As described earlier, 29 yeast strains were selected for evaluation of their capacity to produce gas and biofilm. The biofilm production test on glass slides revealed that the best yeasts were six *P. anomala* and one *G. candidum* strains. *P. fermentans* and *M. guilliermondii* strains demonstrated slightly lower biofilm growth. *K. servazzii* and *T. montevideense* strains were the least efficient for biofilm production, with *T. Montevideense* producing nearly no biofilm (Tab. 3.2). Similar observations were obtained on stainless steel, glass and wood surfaces in past studies as *P. anomala* is well known for its capacity to produce biofilm in the food processing industry (Timke et al., 2008; Didienne et al., 2012; Grounta et al., 2015).

Gas production, which is another undesirable characteristic of the spoilage yeasts, demonstrated more variation. *T. montevideense*, *G. candidum* and *M. guilliermondii* strains produced nearly no gas in all tested conditions. *Pichia* strains produced nearly no gas in sauerkraut juice and cabbage juice at 4 ^{o}C but exhibited a slow production at the optimal temperature, which is not the storage temperature used in the industry. *K. servazzii* strains were the most efficient and exhibited the fastest production of gas in all tested conditions. It was already demonstrated that *P. anomala* has weak spoilage capacities (Timke et al., 2008) in comparison to *K. servazzii*. However, both can have a role of deterioration of the finished food as *K. servazzii* may cause flavor enhancement in specific conditions (Spanoghe et al., 2017).

Strain selection for the CDC Biofilm Reactors analysis was based on specific characteristics: (1) the best strain for producing biofilm with low gas production; (2) the best strain for producing gas with low biofilm growth; and (3) the strain with the best combination of gas production and biofilm growth. The mixed culture was composed of strains selected for characteristics 1 and 2. The selected strains were *P. anomala* strain E147 (1), *K. servazzii* strain E233 (2) and *K. servazzi* strain S40 (3).

Table 3.2 : Results of the preliminary tests for the selection of yeasts strains for their spoilage capacity. The symbol – was used for the absence of gas or biofilm production. The + were used to compare the speed of gas production and the number of viable cells recovered from the biofilm after 72h.

Strains	Species	Gas production in cabbage juice at 27°C	Gas production in cabbage juice at 4°C	Gas production in sauerkraut juice at 27°C	Gas production in sauerkraut juice at 4°C	Biofilm viable cells on glass slides in cabbage juice after 72h at 27°C
E99	*P. anomala*	++	-	++	-	+++
E147	*P. anomala*	++	+ -	+ -	-	+++
E148	*K. servazzii*	+++	+++	+++	+++	+
E150	*P. guillermondii*	+ -	+ -	-	-	++
E166	*P. anomala*	++	+	++	-	+
E167	*P. anomala*	++	-	-	-	+++
E196	*P. anomala*	++	+ -	++	-	+++
E197	*P. anomala*	++	-	-	-	+++
E203	*K. servazzii*	-	-	-	-	+ -
E220	*P. anomala*	+	+ -	++	-	++
E221	*K. servazzii*	+++	++	+++	+++	+
E225	*K. servazzii*	+++	++	+++	+++	+
E228	*P. anomala*	++	+	+	+	+
E229	*K. servazzii*	+++	+	+++	+++	+
E233	*K. servazzii*	+++	++	+++	+++	+
S4	*P. anomala*	++	+ -	+	-	+++
S6	*P. guillermondii*	+ -	-	-	-	++
S39	*K. servazzii*	+++	++	+++	+++	+
S40	*K. servazzii*	+++	+++	+++	+++	+
S51	*T. montevideense*	-	-	-	-	+ -
S54	*P. anomala*	++	+ -	+ -	-	+
S59	*K. servazzii*	++	++	+++	+++	+
S63	*P. anomala*	++	-	+	-	++
S71	*P. anomala*	++	+ -	++	-	+
S72	*K. servazzii*	+++	+++	+++	+ -	+
S86	*P. anomala*	++	+	+++	-	+
S93	*P. anomala*	++	-	+++	-	++
S101	*G. candidum*	-	-	-	-	+++
S159	*P. fermentans*	++	-	+++	+	++

Fermentation of cabbage juice in CDC Biofilm Reactors

Fermentation occurred in all reactors as expected, whether a starter was used or not. When the BLAC I starter culture was used, the pH decreased faster and dropped lower, reaching a mean value of 3.51 pH units when combined to 4 log CFU/ml of yeasts and a mean value of 3.31 pH units when combined with 2 log CFU/ml of yeasts (Tab. 3.3). This difference can be explained by the alcohol production and the competition for the sugars when more yeasts are inoculated since the availability of sugars will influence the quality of the fermentation and therefore the final pH (Fleming et al., 1985; Medina-Pradas et al., 2017). The spontaneous fermentations needed more time to acidify than the starter fermentations and the assays using the starter reached a lower pH, with final pH of 4.06 and 4.33, respectively. Such results are consistent with the literature since, it has been frequently reported that the use of a starter culture allows a better acidification of the medium (Gardner et al., 2001; Johanningsmeier et al., 2007).

Table 3.3 : Evolution of the pH under the different fermentation conditions in CDC Biofilm Reactors with cabbage juice.

Conditions	Yeast strains	pH				
		Day 0	Day 1	Day 4	Day 7	Day 10
4 Log of yeasts + starter culture	E147	5.90	4.89	3.56	3.50	3.49
	E233	5.85	5.00	3.57	3.54	3.47
	S40	5.67	5.16	3.60	3.57	3.50
	E147 + S40	5.81	5.24	3.58	3.55	3.59
2 Log of yeasts + starter culture	E147	5.86	5.09	3.56	3.36	3.36
	E233	5.77	5.13	3.53	3.36	3.26
	S40	5.77	5.08	3.54	3.38	3.29
	E147 + S40	5.89	5.23	3.56	3.41	3.33
4 Log of yeasts + spontaneous fermentation	E147	5.98	5.91	4.15	3.96	3.82
	E233	5.99	5.83	4.01	4.02	4.15
	S40	6.00	5.89	4.22	4.19	4.21
	E147 + S40	6.01	5.88	4.08	4.09	4.05
4 Log of yeasts	E147	5.83	5.82	5.19	4.24	4.11
	E233	5.84	5.77	4.60	4.51	4.51
	S40	5.79	5.75	4.51	4.42	4.44
	E147 + S40	5.92	5.80	4.62	4.37	4.27

Spatial distribution of biofilms in CDC Biofilm Reactors

Figure 3.1 presents the viable yeast cell counts for the 16 conditions, according to the depth of the stainless steel coupons. Results were analysed at top and bottom coupons of each condition separately. Spatial distribution could be observed, since fermentation occurred in a static environment to represent the industrial production of fermented foods. A significant difference between the counts at the surface and the bottom was observed in nearly every trial. When *P. anomala* E147 was used, the variation in the viable counts was between 0.9 and 2.2 log CFU/cm^2, with an average of 1.5 CFU/cm^2, and the counts were always higher at the air-liquid interface (top). *Pichia anomala* reached an average of 7.5 log CFU/cm^2 in biofilms at the surface in comparison with 6.2 log CFU/cm^2 in biofilms at the bottom and was not affected by the presence of *K. servazzii* in mixed culture nor by the presence of LAB starter. Didienne et al. (2012) found a spatial distribution of yeasts in cheese production that would lead to higher biofilm density at the bottom of the wooden gerles used in this study. It was also proposed that a high volume would contribute to such spatial distribution. Therefore, the results in the current study are different from the literature, but this can be explained by the different sites of sampling in the vats, as the other studies used the bottom of the vats instead of the lowest part of the sidewalls.

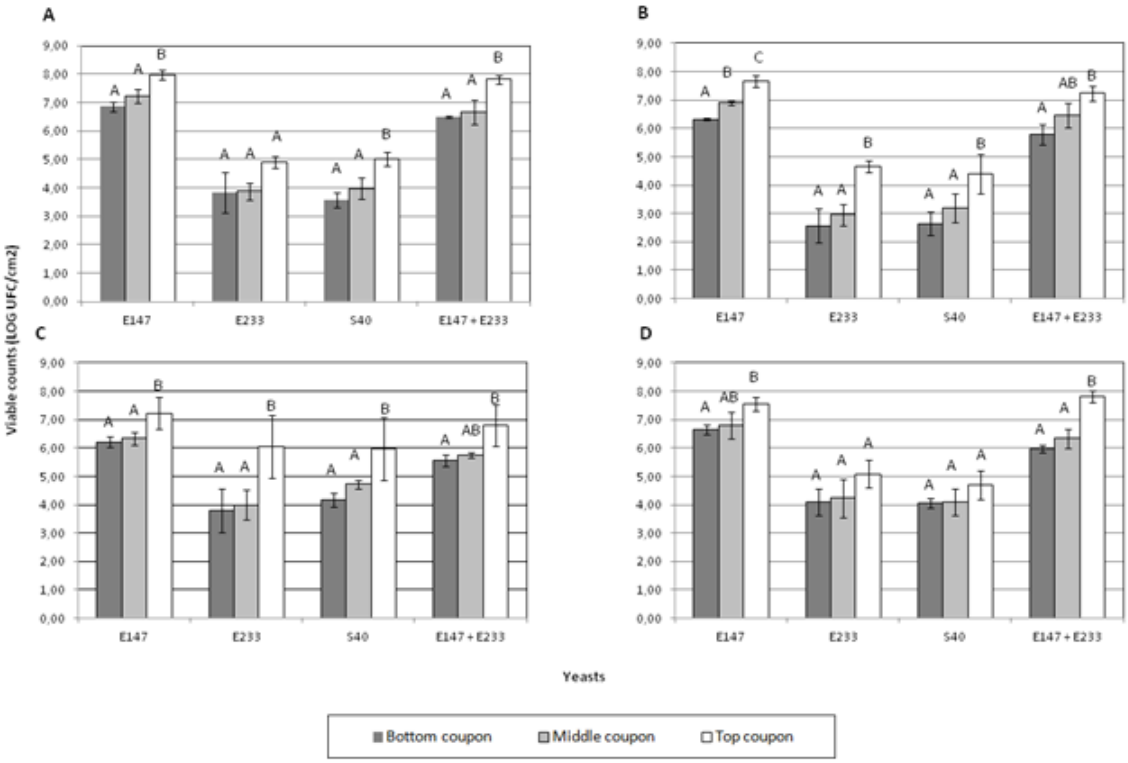

Figure 3.1 : Viable counts of the different yeast strains in biofilm as a function of the coupon's depth in CDC reactors after 10 days at 19°C in different fermentation conditions. An inoculum of 4 log CFU/ml of yeasts with a starter culture in sterile cabbage juice (A), an inoculum of 2 log CFU/ml of yeasts with a starter culture in sterile cabbage juice (B), an inoculum of 4 log CFU/ml of yeasts with a spontaneous fermentation (C) and an inoculum of 4 log CFU/ml of yeasts in sterile cabbage juice (D). Viable counts are the mean of 3 assays and error bars represent standard error on means.

For the pure cultures of *K. servazzii*, less growth was observed, but the difference related to the depth was larger, with counts of 5.1 log CFU/cm^2 observed at the surface versus 3.6 log CFU/cm^2 observed at the bottom. These results suggest a spatial preference for the air-liquid interface (where oxygen is present). This preference can be observed in all the conditions except one: when *K. servazzi* strains were inoculated in pure culture (sterile cabbage juice without starter culture), no significant difference was observed. Three factors may explain this exception. First, the aerobic preference of the yeasts favours their growth at the surface.

Secondly, fermentation by heterofermentative bacteria produces gas that creates a flow towards the surface, leading to a higher concentration of yeasts at the surface early in the fermentation process. Thirdly, the fermentation of LAB could have an increased inhibitory effect on fungal biofilm formation of completely submerged yeasts.

On the other hand, for LAB growth in biofilm, most of the assays demonstrated no significant differences, but variations were clearly observed depending on the depth for three conditions (Fig. 3.2). Those three conditions had in common the use of a starter culture and *P. anomala* E147. Since the yeast biofilm growth was very large in comparison with the bacterial growth, bacteria could have been trapped in the fungal biofilm rather than producing their own biofilm at the surface, which was confirmed by electronic microscopy. These results suggest that bacteria are not influenced by the depth with regard to their surface attachment, in contrast to what was observed with yeasts. These results are in accordance with the literature, as spatial distribution was observed by Grounta et al. (2015) in olives fermentation with plastic vessels.

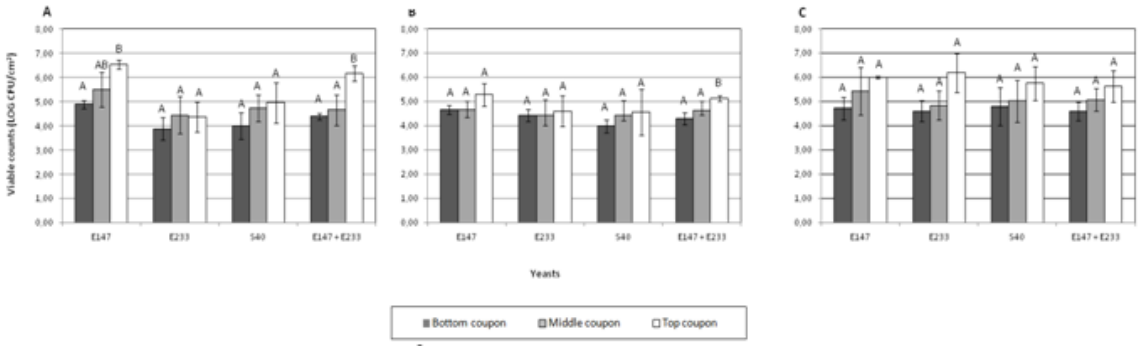

Figure 3.2 : Viable counts of the LAB in biofilm depending on the coupon depth in CDC reactors after 10 days at 19°C in different fermentation conditions with different yeasts. An inoculum of 4 log CFU/ml of yeasts with a starter culture in sterile cabbage juice (A), an inoculum of 2 log CFU/ml of yeasts with a starter culture in sterile cabbage juice (B) and an inoculum of 4 log CFU/ml of yeasts with a spontaneous fermentation (C). Viable counts are the mean of 3 assays and error bars represent standard error on means.

Influence of lactic fermentation on fungal biofilms

Lactic acid bacteria reached their maximum biofilm growth after 4 d with a starter culture, but a small delay was observed in early spontaneous fermentations, independent of the yeast strains. This delay can be explained by the lower inoculum of LAB in spontaneous fermentations (1 log CFU/ml). The stationary phase was reached at a concentration of around 5.0 log CFU/cm^2 (data not shown). Figure 3.3 presents the final counts (after 10 d) of viable biofilm cells for LAB in the three conditions with bacteria. Since no significant differences were found between any of the final LAB counts, it is suggested that LAB from the starter culture or from the indigenous microflora of the cabbage have similar patterns of surface aggregation in cabbage juice. These results suggest that the starter culture would not cause increased biofilm formation on the walls of the vats since the starter did not show more aggregation in comparison with the indigenous microflora. This result is important, because microscopic investigation during other studies observed the formation of a bilayer biofilm where the bacteria would form the first layer followed by the colonization of yeasts (Furukawa et al., 2010).

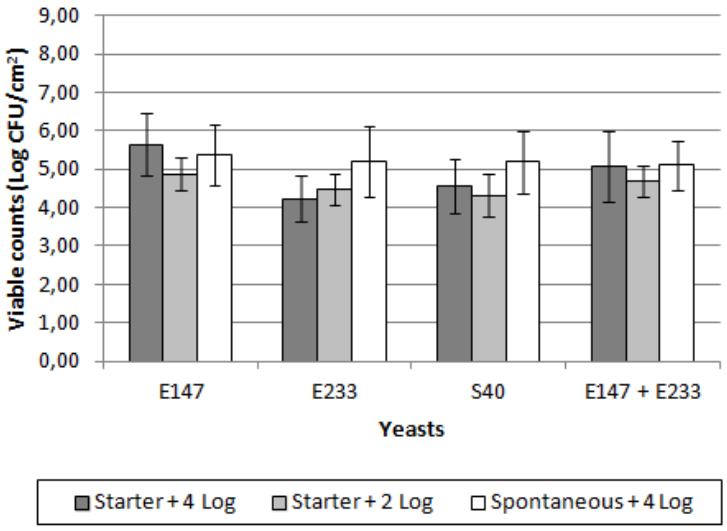

Figure 3.3 : Viable counts of the LAB in biofilm depending on the fermentation conditions after 10 days at 19°C with different yeasts. An inoculum of 4 log CFU/ml of yeasts with a starter culture in sterile cabbage juice, an inoculum of 2 log CFU/ml of yeasts with a starter culture in sterile cabbage juice and an inoculum of 4 log CFU/ml of yeasts with a spontaneous fermentation were tested. Each sample is a mean of 3 coupons on a rod. Viable counts are the mean of 3 assays and error bars represent standard error on means.

Regarding yeasts, *P. anomala* was the best biofilm producer on the glass slides during the preliminary tests and on the stainless steel coupons during the experiment. Figure 3.4 presents the yeast growth in biofilms over 10 d. In most conditions, *P. anomala* reached its maximum growth after 7 d (Fig. 3.4A). The growth curves were similar when a larger inoculum was used but were delayed with a low inoculum level. However, the counts were already similar after 7 d of processing, and no difference was seen after 10 d. A similar pattern was observed for *K. servazzii*, which reached its maximum after 4 and 7 d at high and low inoculums levels, respectively (Fig. 3.4B). Both species had similar growth rates early on after inoculation, but *K. servazzi* reached a stationary phase faster, allowing *P. anomala* to reach higher final concentrations when incubated in pure cultures. There is no census on the time needed for microorganisms to form a mature biofilm as it depends on many factors including the microorganism involved, temperature and media (Brugnoni et al., 2007).

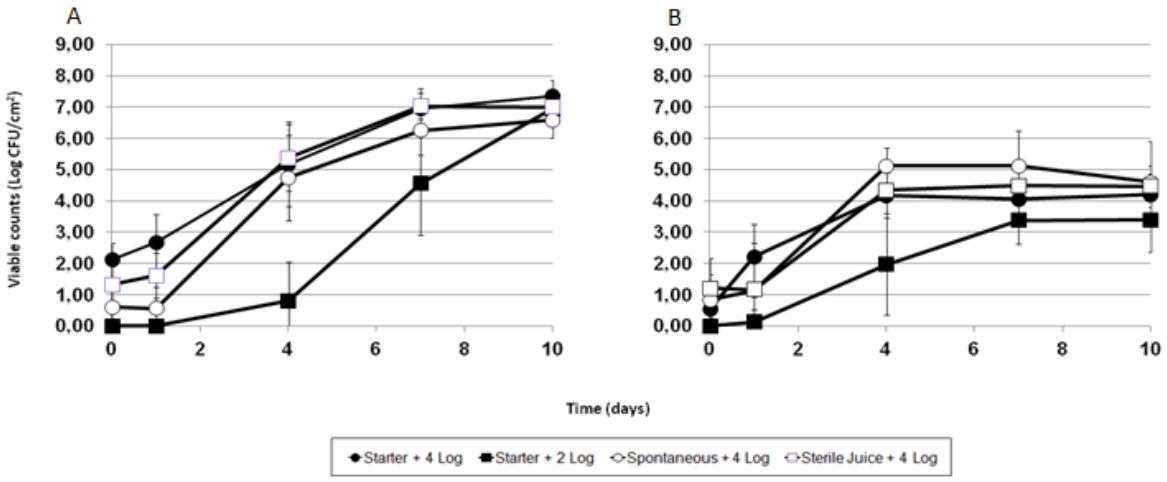

Figure 3.4 : Viable counts of the yeasts in biofilm over 10 days at 19°C in different fermentation conditions for *Pichia anomala* E147 (A) and *Kazachstania servazzii* E233 (B). Viable counts were taken from the 3 coupons on a rod. Results are the mean of 3 assays and error bars represent standard error on means.

One of the main goals of this study was to determine if LAB fermentation could influence fungal biofilm production. Therefore, the final yeast counts in biofilm were compared in the different conditions for the two selected species to the whole-rod counts (mean of the three coupons). For each strain, the results demonstrated no significant differences in final biofilm counts in the different fermentation conditions tested (Fig. 3.5). When analysed separately, no variations were observed between the conditions at the air-liquid interface either (results not shown). Therefore, both the starter culture and the indigenous LAB seem unable to inhibit or even interfere with the growth of acid-tolerant spoilage yeasts in biofilms. Indeed, the main difference observed in yeast growth was related to the species, as *P. anomala* reached 7 log CFU/cm^2 versus 5 log CFU/cm^2 for *K. servazzii* (Fig. 3.5), although a non-significant delay was observed when a starter culture was used. Many studies have demonstrated that mixed microbial cultures could promote the formation of biofilm mainly because of cell communication or quorum sensing (Burmølle et al., 2006; Castonguay et al., 2006; Elias and Banin, 2012). However, in some cases the interaction can be antagonistic because of species competition (RenDueles and Ghigo, 2012; Giaouris et al., 2015). One example is *E. coli* and

Salmonella sp. that could inhibit the formation of biofilm of *Candida tropicalis* (Tarifa et al., 2015). In general, there is a census on one thing and it is that when the environment is harder to live in, the cells have a tendency to grow under biofilm. The growth of cells under biofilm increases significantly the antimicrobial resistance to the environment (Bridier et al., 2011; Brugnoni et al., 2012). This could be one more study arguing that LAB do not influence the production of yeast biofilm, in accordance with most of the literature regarding only the microbial viable counts.

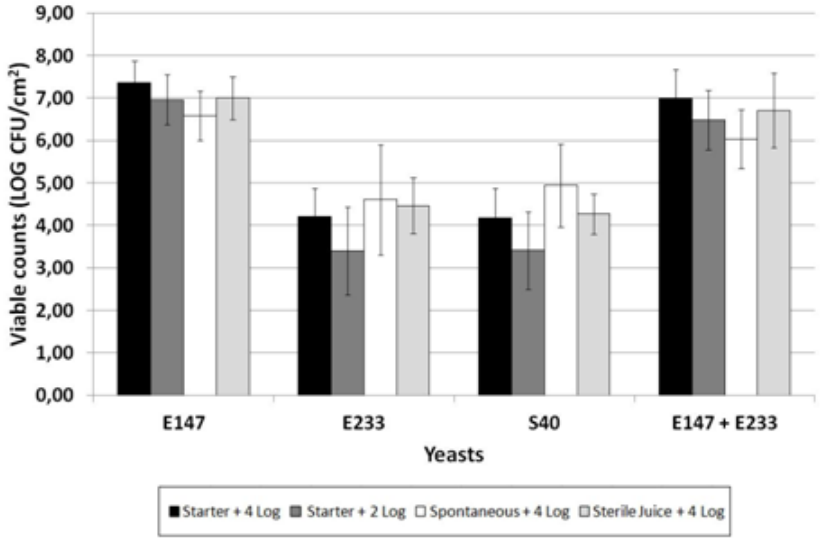

Figure 3.5 : Viable counts of the yeasts in biofilm depending on the fermentation conditions after 10 days at 19°C with different yeasts. An inoculum of 4 log CFU/ml of yeasts with a starter culture in sterile cabbage juice, an inoculum of 2 log CFU/ml of yeasts with a starter culture in sterile cabbage juice, an inoculum of 4 log CFU/ml of yeasts with a spontaneous fermentation and an inoculum of 4 log CFU/ml of yeasts in sterile cabbage juice were tested. Each sample is the mean of 3 coupons on a rod. Viable counts are the mean of 3 assays and error bars represent standard error on means.

Analysis of the planktonic cell counts of LAB showed no differences after 10 d between the use of a starter and the spontaneous fermentation (Fig. 3.6A). Therefore, the BLAC I strain

does not seem to grow in higher concentrations than the indigenous microflora. Moreover, the *K. servazzii* strains showed final viable planktonic cells counts around 6 log CFU/cm^2 in all the conditions tested (Fig. 3.6B). This suggests that the conditions had no impact on the spoilage yeast *K. servazzii*. Those results confirm the benefits of using a starter culture in combination with a killer yeast such as *P. anomala*, as reported by Champagne et al. (2017).

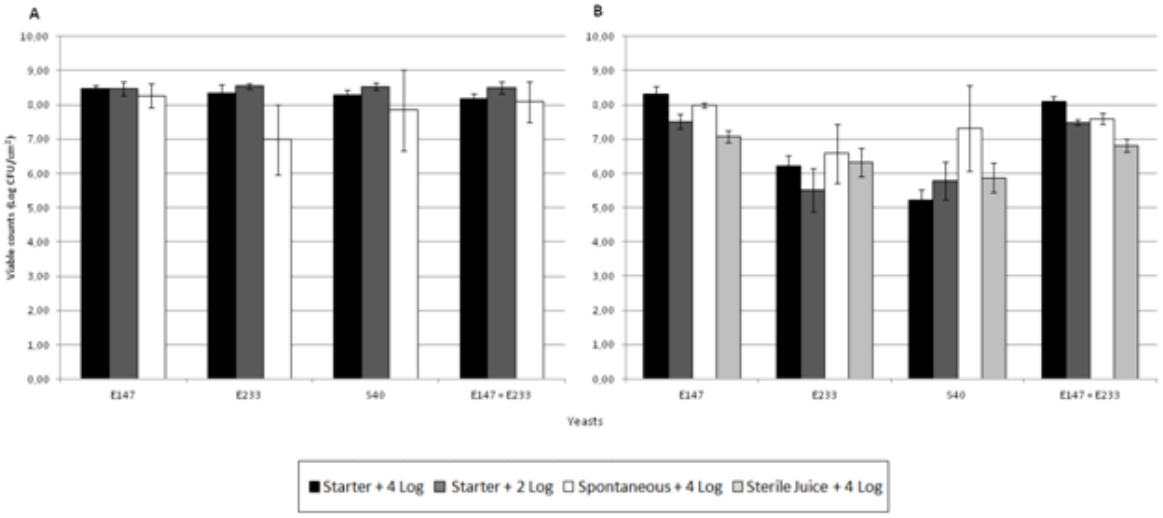

Figure 3.6 : Viable counts of the planktonic cells for LAB (A) and yeasts (B) after 10 days of fermentation of cabbage juice in CDC reactors under different fermentation conditions and yeast strains. Each sample is the mean of 3 coupons on a rod. Viable counts are the mean of 3 assays and error bars represent standard error on means.

Scanning electron microscopy

The observation of the stainless steel coupons with SEM offered more information about the biofilm organization and the distribution of the different microorganisms attached to the surface. In this experiment, the coupons were rinsed with water and then subjected to a chemical wash. Since this was a severe cleaning in comparison with the cleaning used for the

viable counts, more particles that had been weakly bound to the surface were removed. For this section, only the reactors containing the mixed culture of *P. anomala* - E147 and *K. servazzii* - E233 culture were observed in all four conditions with coupons at every depth. It is believed that the other strains would have yielded similar observations.

On the coupons at the bottom, only a few rare yeast aggregates were found and they did not include any bacteria (Fig. 3.7). The main difference between the viable counts and the SEM analysis was observed at this depth. On those coupons, even though growth was detected with the viable counts, no significant biofilm formation could be seen, probably because of the severity of the sample preparation procedures for microscopy compared to the viable counts method. On the middle coupons, small groups of yeasts without specific organization were observed. The middle position offered more variations depending on the conditions. A smaller yeast aggregation was observed on the coupons from the reactors with the BLAC I starter culture than on the coupons with spontaneous fermentation and pure yeast culture (Fig. 3.7). When the yeast inoculum was low (2 log CFU/ml), no yeast attachment was observed on the coupons at the bottom or at the middle of the reactors. At the surface, however, the observed growth was similar to the other conditions with the larger inoculum (Fig. 3.7). The coupons at the air-liquid interface were totally covered by a highly organized and compressed mass of fungal biofilm that hid rare bacteria. Unlike on the submerged coupons, every condition allowed high yeast concentrations on the coupons at the surface. There are only few studies that investigated the formation of mixed biofilms with LAB and yeasts (Kawarai et al., 2007). However, two main conclusions were obtained in those research; first there is no influence from the LAB on the yeast capacity to form biofilm, second, the presence of LAB even promotes the ability of yeast to form biofilm (Hogan and Kolter, 2002; Kawarai et al., 2007; Furukawa et al., 2010; Didienne et al., 2012). Specific combination of bacteria and yeasts can promote the ability of yeast to promote biofilm. There is no census about how the interactions happen between yeast and bacteria during the formation of biofilm, most of the studies consist of trying many combinations to achieve their goal of biofilm promotion between two species to improve the inoculation in the food processing industry.

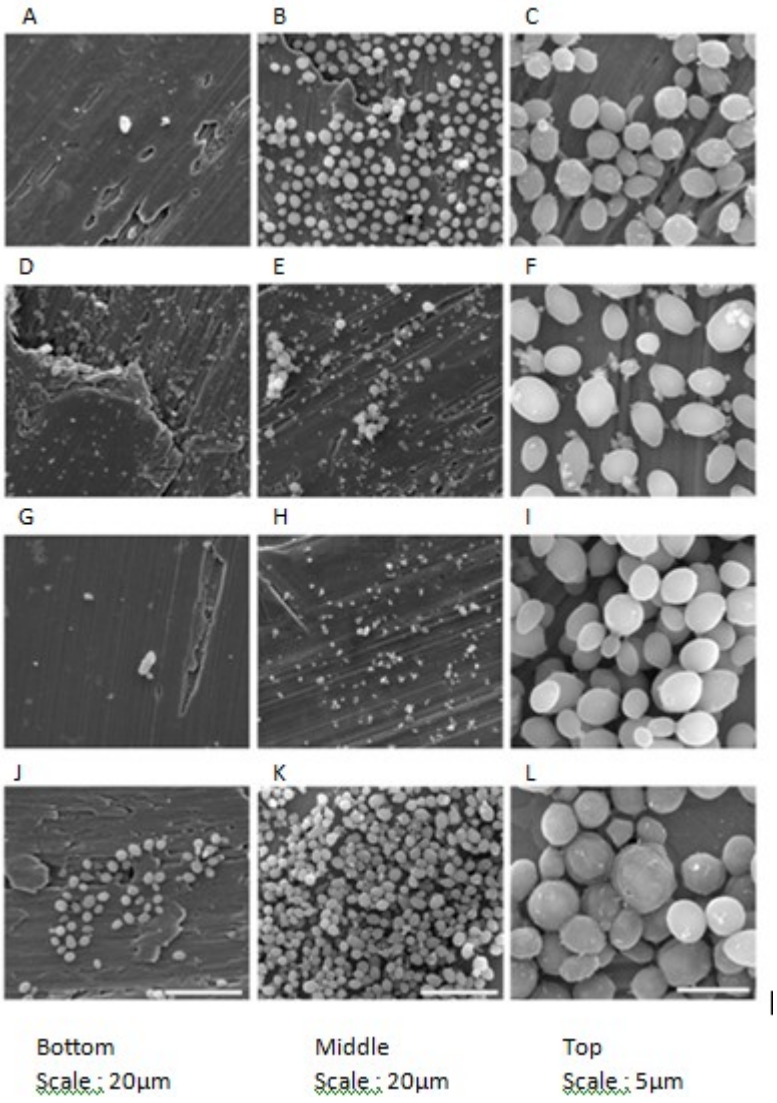

Figure 3.7 : Scanning Electron Microscopy pictures of the coupons after 10 days of fermentation with a co-culture of the yeasts *P. anomala* E147 and *K. servazzii* E233 under different conditions; Spontaneous with 4 log CFU/ml yeasts (A-C), commercial starter with 4 log CFU/ml yeasts in sterile cabbage juice (D-F), commercial starter with 2 log CFU/ml yeasts in sterile cabbage juice (G-I) and sterile cabbage juice with 4 log CFU/ml yeasts (J-L).

Lactic acid bacteria seem to grow principally as planktonic cells instead of producing biofilm. The observation by SEM confirmed that there was a ratio of at least 100 yeasts per bacteria attached to the stainless steel surfaces; even though the planktonic cell counts showed higher LAB numbers, finding bacteria on the coupons by microscopy was a tedious process. It would thus be more appropriate to refer to LAB aggregation on the surface instead of biofilm or simple entrapment. On the other hand, yeasts produced a mature, well organized biofilm at the surface within 10 d but seemed unable to organize their growth in completely submerged conditions, especially in co-culture with the LAB starter. The SEM results were similar to the viable counts but provided more detailed information about the biofilm organization. Since LAB metabolizes the sugars, it can impact the capacity of yeast to produce biofilm (McCourtie and Douglas, 1981; Stepanović et al., 2004). Combined to the fact that fermentation leads to a higher concentration of sugars at the surface, as well as organic acids, could force the yeast to produce biofilm to survive (Tomičić and Raspor, 2017). These observations strongly suggest that the presence of LAB fermentation contributes to the spatial distribution of yeast in biofilms.

CONCLUSION

This research also demonstrated that yeasts isolated from industrial biofilm on the inner walls of stainless steel fermentation vats are able to reproduce biofilm at the laboratory scale. To our knowledge, this is the first report on fungal biofilm in industrial stainless steel fermentation vats. *Pichia anomala* and *Kazakhstnia servazzii*, the two main contaminating yeasts, seem to have developed biofilm on the vats over the years. This could be the main reason for finding the same yeasts in all the vats.

This study has also shown that LAB are not efficient biofilm producers during cabbage juice fermentation and are not affected by the presence of acid-resistant yeasts in the medium.

Moreover, the results of this study suggest that yeast growth would not be affected neither by the presence of LAB at the surface of fermentation vats. However, yeasts seem to produce less dense and less organized biofilm when they are completely submerged and in the presence of LAB. More importantly, it was shown that acid-resistant yeasts contaminating sauerkraut fermentation have a spatial preference for the air-liquid interface. This leads to the hypothesis that LAB could control the yeast contamination if the current fermentation process was adapted to the results of this study.

Although LAB fermentation would be unable to control the growth of acid-resistant yeasts in biofilms at the surface in these conditions, it is believed that a mixed starter composed of LAB and the killer yeast *Pichia anomala* could have interesting potential, as suggested by other studies.

ACKNOWLEDGEMENTS

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DISCUSSION ET CONCLUSION GÉNÉRALE

Ce projet de recherche avait pour but de déterminer les sources de variations dans les fermentations de légumes à l'échelle industrielle qui ont été observées par l'industrie. Les hypothèses investiguées étaient reliées au mode de production des légumes (biologique ou conventionnel) ainsi qu'à l'usage d'un ferment versus la fermentation spontanée. Ensuite, pour déterminer l'origine des problèmes de fermentations secondaires par des levures, l'hypothèse de la présence de biofilms dans les cuves de fermentation a été émise, puis la capacité des levures à produire un biofilm sous des conditions de fermentation lactique a été investiguée.

Le premier objectif de cette étude visait donc à comparer la fermentation de légumes biologiques et conventionnels. Ces deux types de culture ont été comparés par les scientifiques à maintes reprises, mais généralement dans le but de différencier les légumes non transformés. Ces études peuvent être regroupées selon trois principaux aspects La valeur nutritionnelle, la qualité sensorielle et la sécurité alimentaire (Bourn et Prescott, 2002). Selon la croyance populaire, les légumes biologiques seraient plus sécuritaires, auraient meilleur goût, ne contiendraient pas de pesticides et seraient donc plus sains. Toutefois, il n'y aurait aucune variation au niveau de la salubrité des légumes et les légumes produits de manière conventionnelle contiendraient de minimes traces de pesticides (Vannoort et al., 1997; Magkos et al., 2003). Au niveau des aliments fermentés, le principal impact concernerait la microflore indigène du légume, qui peut varier en raison des pesticides, du sol, du climat, du cultivar etc. (El Gindy et al., 1957). Ainsi, il a été supposé qu'il aurait pu y avoir un impact au niveau des fermentations spontanées pouvant être positif ou négatif pour la qualité du produit final, mais sans standardisation. La présente étude n'a toutefois pas permis d'identifier des différences significatives (autres que la mortalité plus lente mais complète des coliformes avec le chou biologique) dans la fermentation du chou qu'il soit conventionnel ou biologique.

Pour éviter la variation apportée par la microflore changeante des légumes, une partie de l'industrie utilise des ferments. Le ferment BLAC I, composé de souches sélectionnées de *Lactobacillus plantarum*, *Leuconostoc mesenteroides* et *Pediococcus acidilactici* a été conçu initialement pour combler des besoins industriels en fermentation des légumes (Gardner et al., 2001). Ce ferment permettait d'obtenir des aliments fermentés de qualité supérieure (acidification, goût, ratio d'acides etc.) ne nécessitant pas de pasteurisation. Bien que l'utilisation des ferments soit peu commune dans l'industrie des légumes, le ferment BLAC I est commercialement utilisé et vendu au détail depuis plus de 20 ans. Ce ferment est présentement utilisé par plusieurs entreprises et sert à la production de divers aliments fermentés comme le kimchi et la fleur d'ail, mais principalement pour la choucroute, la carotte et la betterave. La fermentation avec le ferment mixte BLAC I permettait, au moment de sa mise au point, de contrôler la fermentation en plus de réduire au minimum les sucres fermentescibles résiduels. La présence de ces sucres durant la phase de maturation pourrait générer des fermentations secondaires par des levures acido-résistantes résultant à la production de gaz (CO_2) et à la consommation des acides organiques qui assurent la salubrité des aliments fermentés (Breidt et al., 2013b).

Le projet a permis de valider l'efficacité du ferment actuel pour l'obtention d'un produit de fermentation de qualité supérieure à la fermentation spontanée grâce à une acidification plus rapide, démontrant cependant une mortalité hâtive au cours de la phase de maturation, ce qui n'est pas le cas en fermentation spontanée, bien que le résultat final après six mois soit le même. Il a aussi été démontré que l'évolution de la microflore indigène du chou au cours des dernières décennies n'aurait pas d'influence sur la qualité d'une fermentation avec l'usage d'un ferment. Toutefois, *Lactobacillus brevis* a été détecté en fin de fermentation malgré l'usage du ferment au cours de cette étude. Cette bactérie lactique, étant connue pour être plus résistante aux acides organiques comparativement à *Lactobacillus plantarum*, représente une avenue intéressante pour améliorer la survie du ferment et pourrait éventuellement être ajoutée à ce dernier (Medina-Pradas et al., 2017). Les résultats ont aussi démontrés que la fermentation lactique du chou, qu'elle soit opérée avec ou sans ferment (spontanée), permet la

croissance de levures acido-résistantes à la surface des fermentations lors des productions en cuves industrielles, ce qui n'a pas été observé à l'échelle laboratoire et qui suggère donc la présence de biofilms fongiques.

Les biofilms représentent un problème récurrent dans l'industrie alimentaire car ils génèrent un risque au niveau sanitaire par la persistance de bactéries pathogènes, ainsi que des pertes industrielles en raison des microorganismes dégradant ou altérant les différents aliments. L'industrie de la transformation alimentaire est touchée à tous les niveaux, que ce soit en transformation des viandes et de légumes ou en production de bière, de fromage et autres (Timke et al., 2008; Cloete et al., 2009; Srey et al., 2013). Toutefois, dans certains cas, la présence de cellules persistantes peut avoir un rôle de ferment, inoculant le lait en production fromagère par exemple (Didienne et al., 2012). Afin de réduire la capacité des microorganismes à adhérer aux surfaces dans les usines, l'acier inoxydable est devenu le matériau le plus utilisé (Myszka et Czaczyk, 2011; Moreira et al., 2015). Il a été démontré qu'il était plus difficile pour les microorganismes de produire un biofilm sur cette surface hydrophile, variant toutefois selon le fini du matériau (Arnold et Bailey, 2000). Malgré ces connaissances, il est surprenant de voir l'apparition de biofilms dans les cuves de fermentation de choucroute indépendamment de l'usage de l'acier inoxydable et de la présence d'un milieu très difficile pour la croissance microbienne par sa grande acidité, la présence d'acides organiques et la croissance de bactéries lactiques pouvant compétitionner avec les levures.

Les levures *K. servazzii* et *P. anomala* ayant été retrouvées dans les choucroutes ont aussi été isolées à la surface interne des cuves de fermentation, confirmant la présence de biofilms. C'est pourquoi la capacité de certaines souches de levures à former des biofilms sur des coupons en acier inoxydable a été évaluée dans des conditions de fermentation de jus de chou en réacteurs CDC à l'échelle laboratoire. Ceci a permis de confirmer que les levures contaminant le procédé pouvant produire des biofilms sur ce type de surface et ce même en

présence du ferment. L'étude a aussi démontré une préférence spatiale des levures pour la formation de biofilms à l'interface air-liquide.

Cette étude apporte des pistes de solutions à l'industrie afin de modifier les procédés actuels pour inhiber complètement la croissance des levures ainsi que pour améliorer le ferment. La mise en place d'un nouveau procédé de fermeture des cuves ainsi que l'utilisation d'une levure killer seraient des solutions à investiguer afin de remédier aux problèmes de biofilms et de contamination fongique lors de la phase de maturation. L'ajout de *Lactobacillus brevis* dans un ferment pourrait permettre d'améliorer la survie des bactéries lactiques à long terme lors de la phase de maturation de la choucroute.

Dans toute la complexité de l'étude des biofilms, très peu de recherches ont examiné le développement de biofilms selon la hauteur dans les cuves de fermentations, que ce soit au niveau de légumes, produits laitiers ou produits alcoolisés. Ainsi, cette étude a su démontrer qu'il y avait un intérêt à étudier ce genre de variations au niveau du développement de biofilms dans l'industrie alimentaire.

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